Functional analysis of the butyrolactone autoregulator receptor (FarA) involved in the secondary metabolism of *Streptomyces lavendulae* FRI-5

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Shigeru Kitani

Department of Biotechnology, Graduate School of Engineering,

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(放線菌 *Streptomyces lavendulae* FRI-5 の二次代謝生産制御機構における
オートレギュレーターリセプターの機能解析)

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Shigeru Kitani

Department of Biotechnology, Graduate School of Engineering,

Osaka Univeristy

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Chapter 1

General introduction

The filamentous, Gram-positive bacterial genus *Streptomyces* strains are widespread in nearly all natural environments, including land, sea, river, and the atmosphere. Soil is the most popular habitat of streptomycetes, and the frequency of their isolation from the soil is extremely high in comparison with other bacterial taxa. It is well known that members of the genus *Streptomyces* produce a great many antibiotics and other classes of biologically active secondary metabolites. Actinomycetes do indeed make up some two-thirds of the known antibiotics produced by microorganisms (some definitions actually restrict the word "antibiotic" to microbial products), and amongst them nearly 80% are made by members of the genus *Streptomyces*, with other genera trailing numerically: *Micromonospora* is the runner-up with fewer than one-tenth as many as *Streptomyces* (Table 1.1). If secondary metabolites with biological activities other than anti-microbial are included, actinomycetes are still out in front, making up over 60% of known species (again *Streptomyces* species account for 80% of these). As such, the genus *Streptomyces* is the most valuable producer of biologically active compounds useful to both humans and animals.

Table 1.1 Approximate numbers of secondary metabolites produced by different groups of organisms, as of 1994 (Kieser *et al.*, 2000).

Source	Bioactive metabolites			"Inactive" metabolites
	Antibiotics	Other	Total	
Non-actinomycete bacteria	1400 (12%)	240 (9%)	1640 (11%)	2000-5000
Actinomycetes	7900[1] (66%)	1220[1] (40%)	9120[1] (61%)	8000-10000
Fungi	2600 (22%)	1540 (51%)	4140 (28%)	15000-25000
Total microorganisms	11900 (100%)	3000 (100%)	14900 (28%)	25000-40000
Lichens	150	200-500		~1000
Algae	700	800-900		1000-2000
Higher plants	5000	25000-35000		500000-800000
Terrestrial animals	500	10000-15000		200000-300000
Marine animals	1200	1500-2000		2000-3000
Total higher organisms	7500	35000-50000		>1000000

[1] In each category, nearly 80% were found in *Streptomyces* and 20% in other actinomycetes

1.1 Two physiological features of streptomycetes.

Two physiological features are characteristic of all streptomycetes: (1) production of secondary metabolites, including antibiotics; and (2) a complex life cycle of morphological differentiation. These features are briefly described below.

Physiology and regulation of antibiotic production.

Antibiotic production in streptomycetes is generally growth phase-dependent. In liquid culture, production begins as the culture enters the stationary phase. In agar-grown cultures, it coincides with the onset of morphological differentiation, and the occurrence of mutants deficient in both antibiotic production and the formation of aerial hyphae indicates at least some common elements of genetic control (Chater and Bibb, 1997). Most antibiotics are the products of complex biosynthetic pathways, with a

cluster of genes (generally 20-30) being dedicated to the synthesis of any one compound. These gene clusters usually contain pathway-specific regulatory genes that act as transcriptional activators, and that are themselves subject to control by pleiotropic regulatory genes. The onset of antibiotic biosynthesis is determined and influenced by a variety of physiological and environmental factors. These include growth rate, imbalances in metabolism (Hood *et al.*, 1992), various physiological stresses (Hobbs *et al.*, 1992; Yang *et al.*, 1995), and diffusible γ-butyrolactone signaling molecules called "γ-butyrolactone autoregulators" (Horinouchi and Beppu, 1994). In addition to these positive effectors of antibiotic production, antibiotic synthesis may also be subject to metabolite repression and/or inhibition by readily utilized sources of nitrogen (generally NH_{4+}), phosphate, and/or glucose (Demain, 1992; Demain and Fang, 1995; Chater and Bibb, 1997). The various factors influencing antibiotic production in streptomycetes are not readily accommodated in a simple unifying model. However, it seems reasonable to propose an overall regulatory influence over the growth rate, with superimposed pathway-specific regulatory effects influencing the production of individual antibiotics. These effects may be elicited at the level of expression of the pleiotropic or pathway-specific regulators or the biosynthetic structural genes, and/or at the level of activity of the biosynthetic enzymes.

Most genetic studies of antibiotic regulation have used *S. coelicolor* A3(2), which produces at least four different antibiotics, permitting studies of pathway-specific and pleiotropic control of antibiotic production to be carried out. Over 20 different pleiotropic genes influence antibiotic production in *S. coelicolor* (Bibb, 1996; Chater and Bibb, 1997), several of which (the *bld* genes) are also required for sporulation. While the roles of most of these genes are unknown, they include two component regulatory systems and eukaryotic-like serine-threonine protein kinase, indicating an important role for

protein phosphorylation in the regulation of antibiotic production. In particular, little is understood of the signals that trigger the onset of antibiotic production and that presumably activate expression of some or all of these pleiotropic regulatory genes. These genes are likely to form a complex regulatory web, consistent with the need of the organism to sense, integrate, and respond to a variety of environmental and physiological signals. Recently, one of these signals in *S. coelicolor* has been found and identified to be a γ-butyrolactone autoregultor called "SCB1" (*Streptomyces coelicolor* butyrolacotne autoregulator), and its function in antibiotic production has been analyzed (Takano *et al.*, 2000). This phenomenon is described in more detail in the next section (**1.2**).

Complex life cycle of morphological differentiation.

One of the most striking characteristics of the *Streptomyces* species is a complex cycle of morphological differentiation such as the germination of spores, the elongation and branching of substrate mycelia, the formation of aerial mycelia, and the septation of hyphae and spore maturation (Fig. 1.1; Chater, 1993). As colonies grow, the older mycelium forms dense piles, and further changes take place, changes that may be responses to nutrient limitation or other physiological stresses (some perhaps mediated via ppGpp: Ochi, 1986; Takano and Bibb, 1994; Chakraburtty and Bibb, 1997) and/or to cell density via extracellular signals (examples include A-factor in *S. griseus*, described in the next section (**1.2**)) and an apparent cascade of signals necessary for aerial growth in *S. coelicolor* A3(2) (Willey *et al.*, 1993). The changes are of at least five general kinds: increased production of some extracellular mycelium; an onset of secondary metabolism; initiation of lysis of some compartments of the substrate mycelium; an onset of storage metabolism in substrate hyphae on the colony surface; and growth initiation of aerial hyphae. Some of the *bld* genes appear to play a role in several of the above. For

example, *bldA* mutants in *S. coelicolor* A3(2)-depending on growth conditions-may lack aerial growth, antibiotic production, and glycogen (Plaskitt and Chater, 1995).

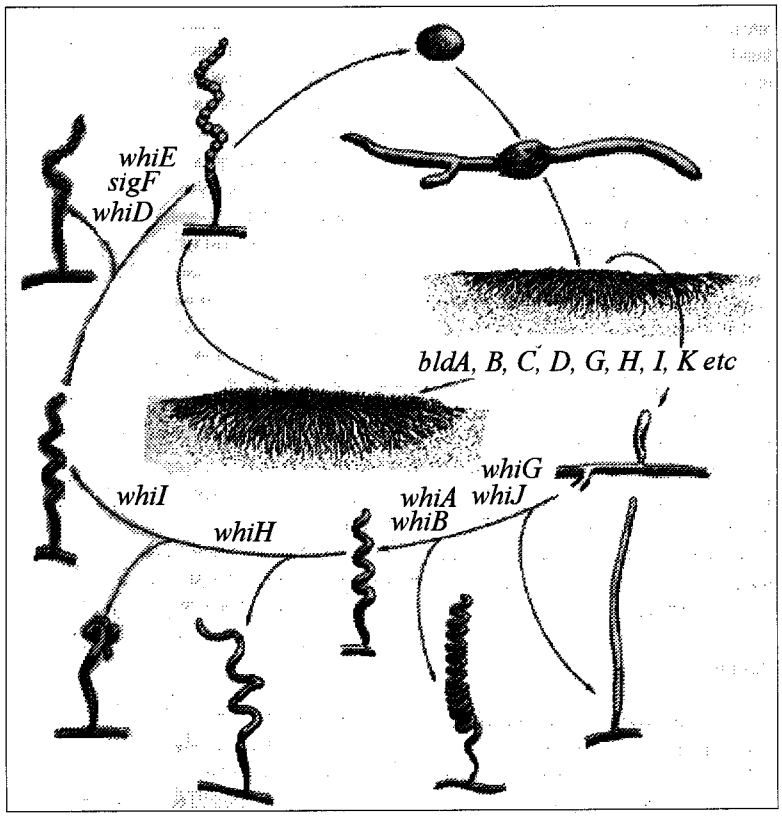

Fig. 1.1 The life cycle of *Streptomyces coelicolor* A3(2). Under favourable conditions, one or two germ tubes emerge from a spore and grow by tipextension and branch formaiton to give rise to a substrate mycelium. After about 2-3 days, aerial hyphae grow up in a process that involves the action of a large number of *bld* genes. The apical compartment of individual aerial hyphae forms a spiral syncytium that contains many tens of genomes. When aerial growth stops, multiple septa subdivide the apical compartment into unigenomic pre-spore compartments. These subsequently change in shape; wall thicking occurs and grey spore pigment is deposited, to generate dessiccation-resistanst spores. Sporulation septation depends on six regulatory loci (*whiA, B, G, H, I* and *J*), which are also needed for expression of at least one (*sgiF*) of two regulatory loci, *whiD* and *sigF*; these in turn play importants roles in the thicking of the spore wall and the deposition of the grey spore pigment (spore pigment is specified by the *whiE* gene cluster). The terminal phenotypes of mutants blocked at different stages, shown here by side-arrows before the relevant genes, are somewhat different from intermediate stages of normal sporulation.

Remarkably, *bldA* specifies the only tRNA responsible for the leucine codon UUA, which (as TTA) is particularly rare in the high G+C DNA of streptomycetes but is often present in pathway-specific regulatory genes for antibiotic biosynthesis (Leskiw *et al.*, 1991).

Streptomyces colonies are multicellular and differentiated organisms exhibiting temporal and spatial control of gene expression, morphogenesis, metabolism, and the flux of metabolites. An extensive knowledge of the developmental biology of streptomycetes is not only of basic scientific interest, but also provides a valuable resource for optimizing their industrial exploitation, whether through a fuller understanding of the genetic interconnections of development and secondary metabolism, and of metabolic routing and re-routing, or in the preparation of inocula or controlling the growth form in fermenters.

1.2 γ-Butyrolactone autoregulators and its cascade in *Streptomyces* species.

Among the factors known to affect antibiotic production and/or morphological differentiation in streptomycetes, γ-butyrolactone autoregulators (Fig. 1.2) have been shown in several streptomycetes to serve as extracellular signaling molecules determining the onset of the two noteworthy characteristics. Thus far, 10 butyrolactone autoregulators have been isolated that are very widely distributed among useful industrial *Streptomyces* species (Hashimoto *et al.*, 1992). These autoregulators have a common 2,3-disubstitued γ-butyrolactone skeleton and can be classified into three groups based on minor structural differences. To clarify the autoregulator-dependent regulation mechanism for antibiotic production and/or morphological differentiation, much research on various aspects of regulation have been carried out. These studies are briefly described below:

IM-2 [(2R, 3R, 1'R)-2-(1'-hydroxybutyl-3-hydroxymethyl) γ-butanolide]

IM-2 of *Streptomyces lavendulae* FRI-5 triggers the production of the nucleoside antibiotics showdomycin and minimycin as well as an undefined blue pigment at a concentration of only 3 nM at the stationary phase (Fig. 1.3; Hashimoto *et al.*, 1992;

Mizuno *et al.*, 1994; Sato *et al.*, 1989; Yanagimoto *et al.*, 1983). During the same phase, the production of the antituberculosis antibiotic D-cycloserine is terminated.

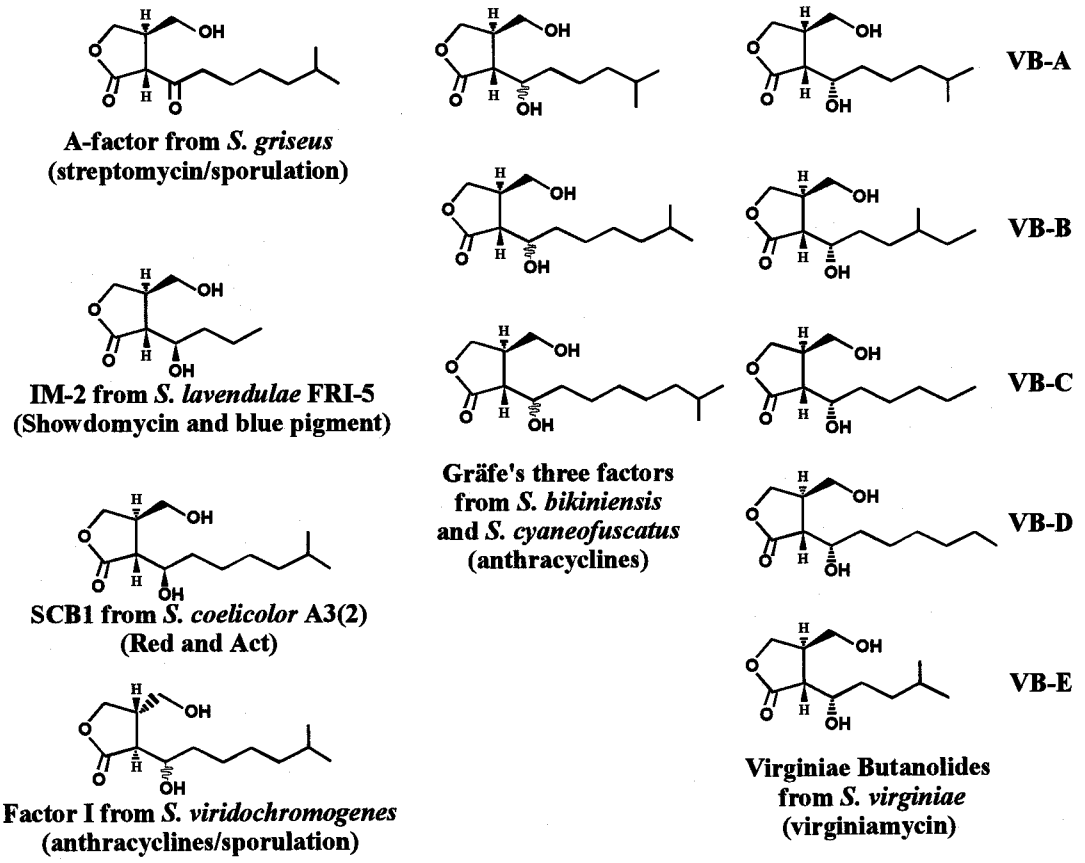

Fig. 1.2 Structures of γ-butyrolactone autoregulators from *Streptomyces* species. Absolute configurations of A-factor (Kleiner *et al.*, 1976; Mori, 1983), VBs (Yamada *et al.*, 1987; Kondo *et al.*, 1989; Sakuda and Yamada, 1991), IM-2 (Sato *et al.*, 1989) and SCB1 (Takano *et al.*, 2000) have been assigned to (3R), (2R, 3R, 1'S), (2R, 3R, 1'R) and (2R, 3R, 1'R), respectively, as shown. Absolute configurations of Factor I and three factors from *S. bikiniensis* and *S. cyaneofuscatus* are not yet detemined (Gräfe *et al.*, 1982;1983)

The phenomenon in which IM-2 exerts both positive and negative effect is unique, as all other autoregulators as described below have only positive effects on antibiotic production or morphological differentiation. The IM-2 specific receptor protein (FarA; FRI-5 autoregulator receptor A, 25 kDa) has been purified (Ruengjitchatchawalya *et al.*, 1995), and the corresponding gene (*farA*) has been cloned, sequenced, and analyzed (Waki *et al.*, 1997). These analyses have revealed a helix-turn-helix (HTH) DNA binding motif in the

N-terminus of FarA, an IM-2-dependent increase in *farA* transcription, and IM-2-specific ligand binding of both native FarA and recombinant FarA expressed in *E. coli*, indicating that FarA may regulate the transcription of genes involved in the biosynthesis of secondary metabolites in an IM-2-dependent manner.

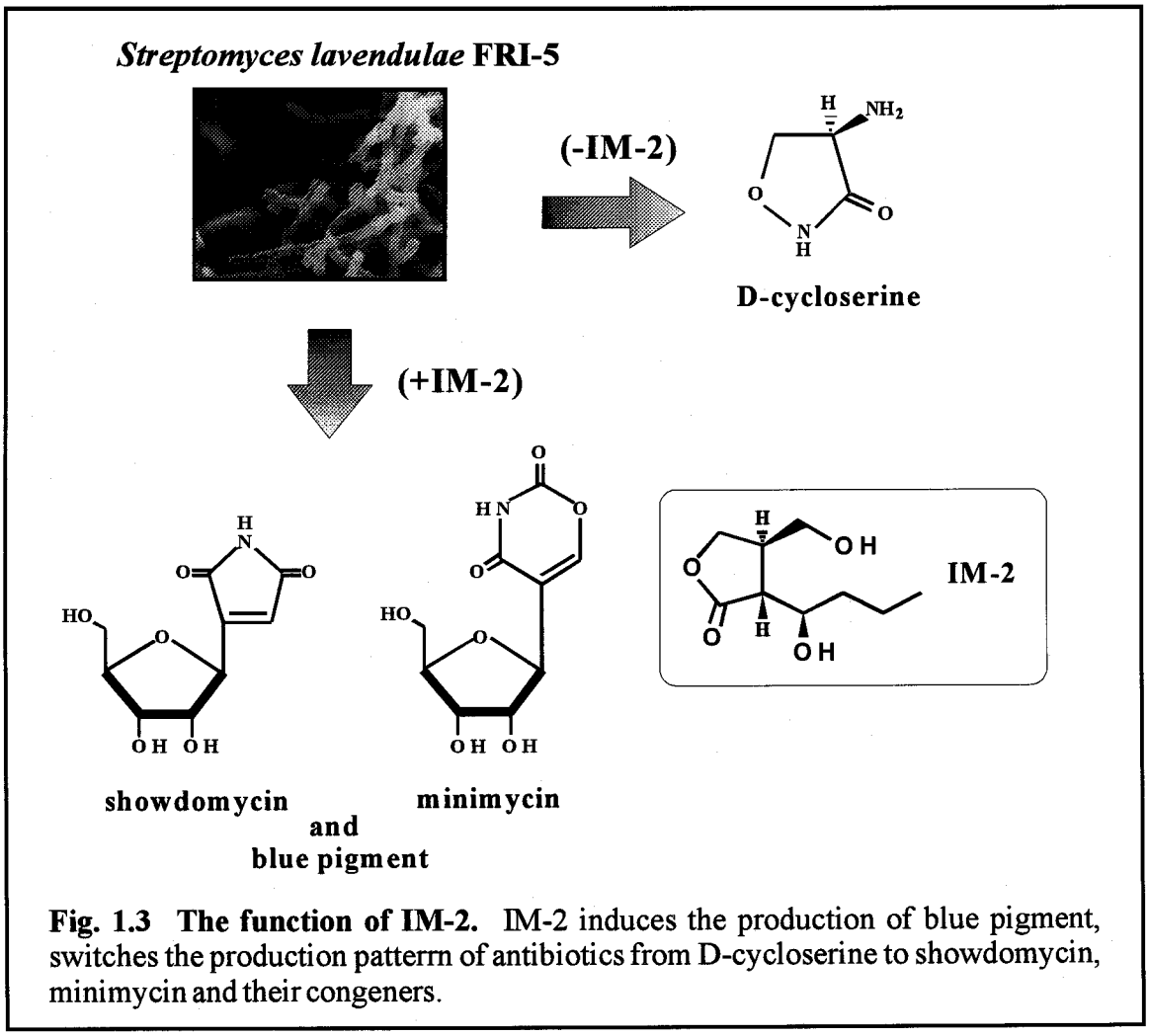

Fig. 1.3 The function of IM-2. IM-2 induces the production of blue pigment, switches the production pattern of antibiotics from D-cycloserine to showdomycin, minimycin and their congeners.

A-factor [(2R, 3R)-2-(isocapry-3-hydroxymethyl) γ-butanolide]

A-factor was the first butyrolactone autoregulator identified and found in the culture broth of *Streptomyces griseus*, a streptomycin producer. A-factor, which is the most extensively studied of the autoregulators (Hara and Beppu, 1982; Khoklov, 1988; Khoklov *et al.*, 1967), triggers both aerial mycelium formation and streptomycin production at an extremely low concentration (Horinouchi and Beppu, 1992; 1994; Horinouchi, 1996).

Recently, a regulatory cascade leading to streptomycin production and morphological differentiation was almost revealed (Fig. 1.4; Ohnishi *et al.*, 1999; Yamazaki *et al.*, 2000). When A-factor is gradually accumulated in the culture in a growth dependent manner and the concentration reaches a certain critical level, it binds to ArpA (A-factor receptor protein A) (Onaka *et al.*, 1995).

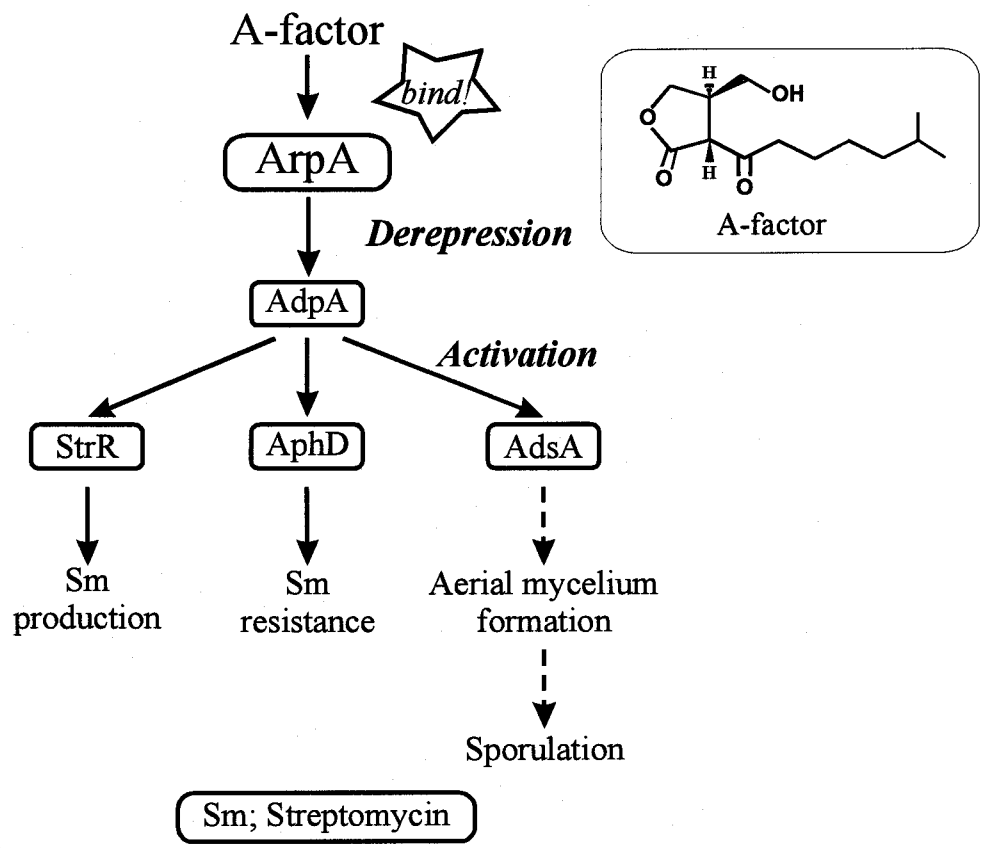

Fig. 1.4 Pleiotropic regulatory system of streptomycin production, self-resistance and sporulation by A-factor. The A-factor signal is transmitted to StrR, AphD and σ^{AdsA} via ArpA and AdpA. One or more genes controlled by AdpA should be involved in aerial mycelium formation since introduction of *adsA* into Δ*adpA* strains does not restore aerial mycelium formation. AdpA also activates *strR* encoding a pathway specific transcriptional activator for streptomycin biosynthetic genes.

ArpA has been purified and the corresponding gene (*arpA*) cloned by a group at Tokyo University in cooperation with Prof. Yamada's laboratory of Osaka University. ArpA binds to the promoter of *adpA* (A-factor-dependent protein A) and represses its transcription (Ohnishi *et al.*, 1999). ArpA is released from this promoter region by

binding with A-factor. This derepresses the *adpA* transcription and the expressed AdpA protein then activates transcription of *strR* by binding to an upstream activation sequence (Vujaklija *et al.*, 1991; 1993). A pathway-specific transcriptional activator, StrR, activates transcription of most of the streptomycin biosynthetic genes by binding multiple sites in the gene cluster (Distler *et al.*, 1992; Retzlaff and Distler, 1995). This also activates *strR*-cotranscribed *aphD* encoding streptomycin-6-phospho-transferase as self-resistance machinery (Vujaklija *et al.*, 1991; 1993). As for the triggering mechanism of morphological differentiation, AdpA is also involved by regulating the transcription of the extracytoplasmic-function-sigma-factor *adsA* gene that is concerned with aerial mycelium formation (Yamazaki *et al.*, 2000). This cascade is very simple with respect to regulation; specifically ArpA represses the transcription of *adpA*, the principle regulator in streptomycin production and morphological differentiation.

VBs (Virginiae butanolides)

[(2R, 3R, 1'S)-2-(1'-hydroxyalkyl-3-hydroxymethyl) γ-butanolide]

VBs consist of VB A, B, C, D, and E, (Fig. 1.2), all of which induce the production of viginiamycin [composed of two types of antibiotics; viginiamycin M_1 (VM_1) and virgniamycin S (VS)] at nM concentrations in *Streptomyces virginiae* (Yamada *et al.*, 1987; Kondo *et al.*, 1989). BarA (γ-butyrolactone autoregulator receptor A) was isolated as a mediator of the VB signal in our laboratory (Okamoto *et al.*, 1985), and a regulatory cascade via BarA has been characterized in vitro and in vivo (Fig. 1.5; Kinoshita *et al.*, 1997; 1999; Nakano *et al.*, 1998; 2000). These analyses have revealed that BarA is a DNA-binding transcriptional repressor, and that two of the target genes (*barB* and the VS-specific transporter *varS*) are located immediately downstream of the *barA* gene, indicating that the antibiotic resistance mechanism is also regulated by BarA in *S.*

virginiae (Lee *et al.*, 1998). BarA forms an autoregulatory circuit, which should serve to sense and maintain intracellular free VB concentrations, by binding to its own promoter in the absence of VB and dissociating from it in the presence of VB (Kinoshita *et al.*, 1997; 1999). Phenotypic analysis of an in-frame deletion mutant and a null mutant of *barA* also provided in vivo evidence that the VB-BarA pathway participates not only in virginiamycin production but also in autoregulator biosynthesis (Nakano *et al.*, 1998; 2000). However, the overall regulatory cascades via VB in secondary metabolism still remains unclear in comparison with the A-factor cascade.

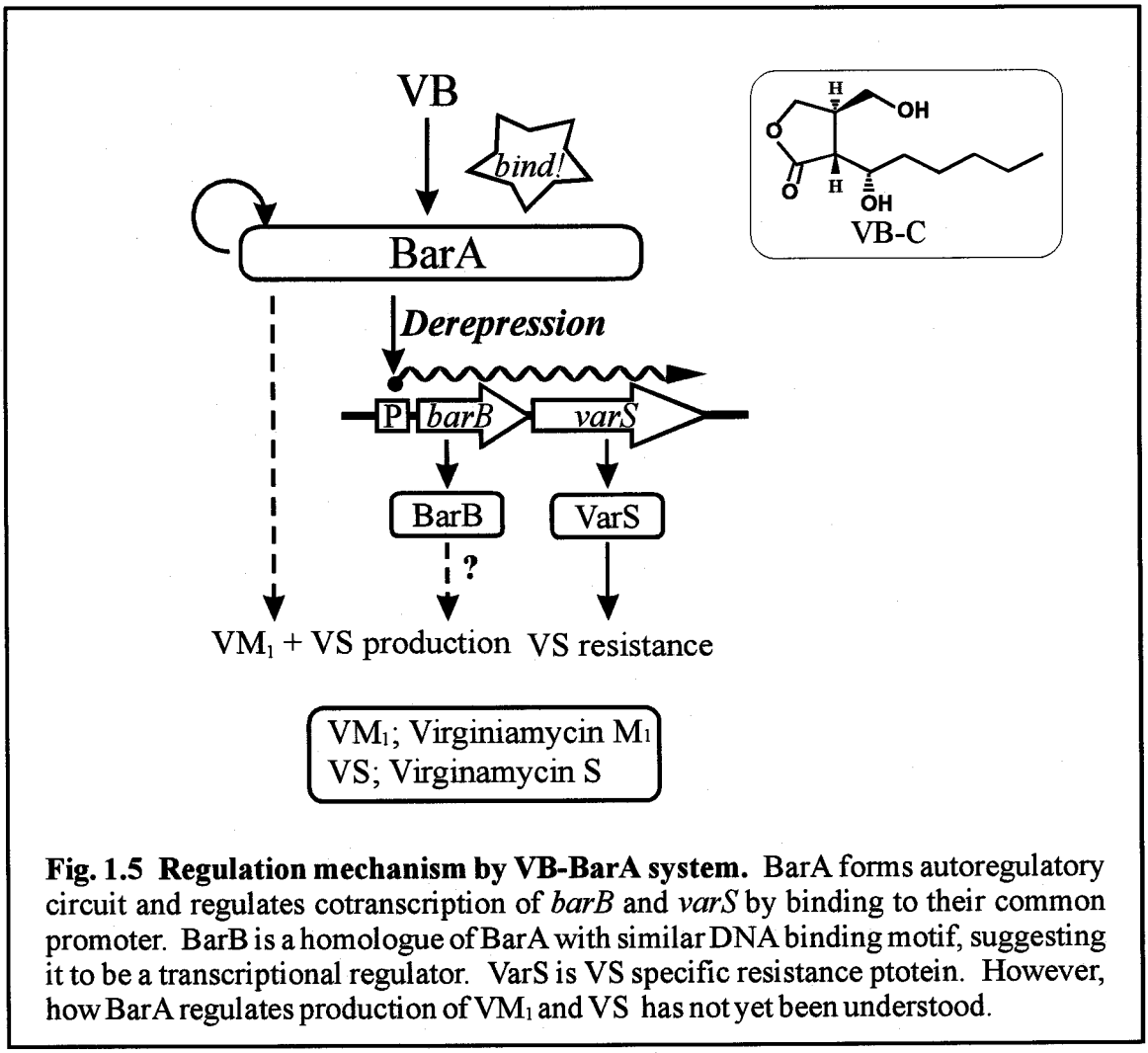

Fig. 1.5 Regulation mechanism by VB-BarA system. BarA forms autoregulatory circuit and regulates cotranscription of *barB* and *varS* by binding to their common promoter. BarB is a homologue of BarA with similar DNA binding motif, suggesting it to be a transcriptional regulator. VarS is VS specific resistance ptotein. However, how BarA regulates production of VM_1 and VS has not yet been understood.

SCB1 (*Streptomyces coelicolor* γ-butyrolactone autoregulator 1)

[(2R, 3R, 1'R)-2-(1'-hydroxy-6-methylheptyl-3-hydroxymethyl) γ-butanolide]

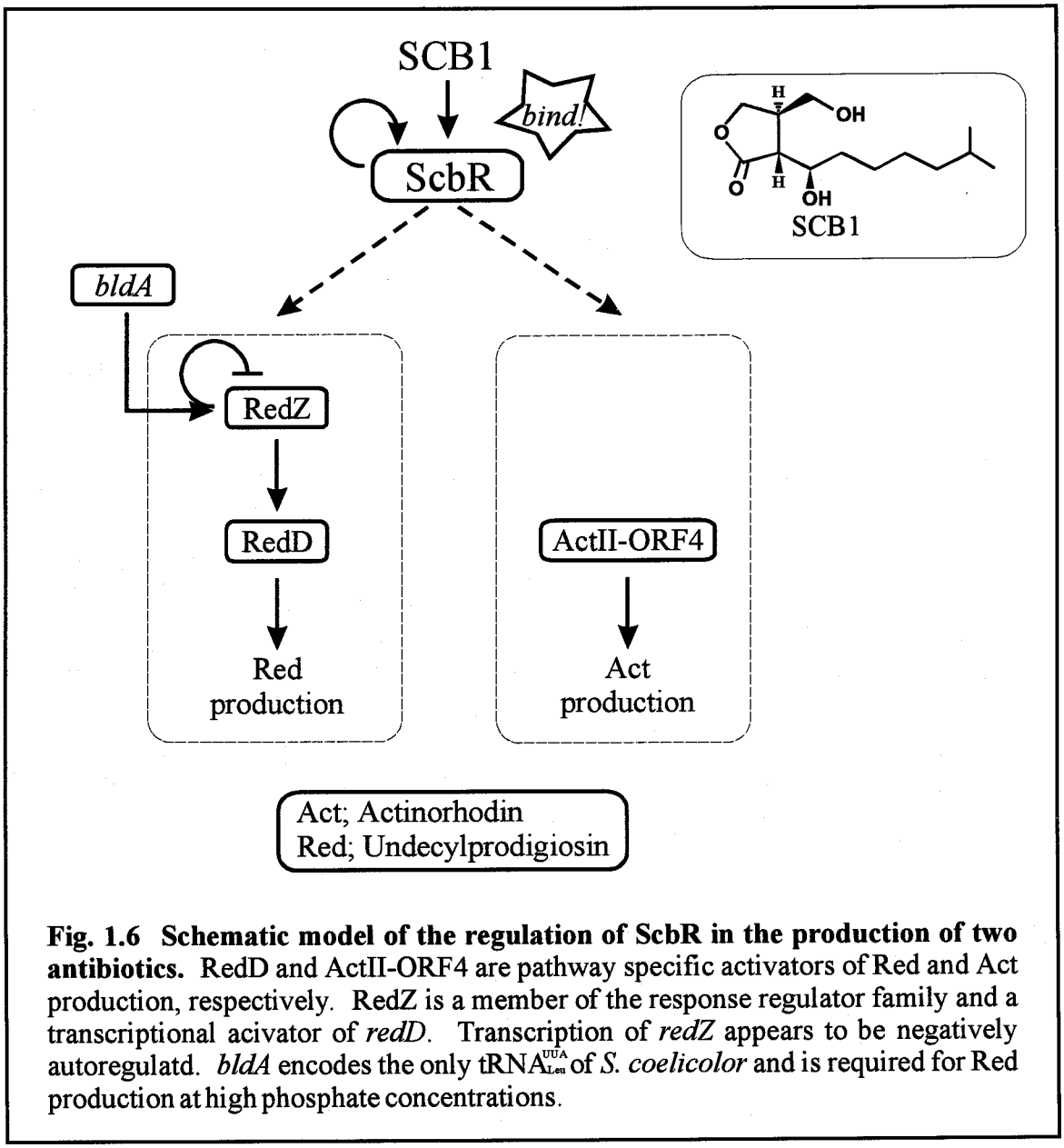

Fig. 1.6 Schematic model of the regulation of ScbR in the production of two antibiotics. RedD and ActII-ORF4 are pathway specific activators of Red and Act production, respectively. RedZ is a member of the response regulator family and a transcriptional acivator of *redD*. Transcription of *redZ* appears to be negatively autoregulatd. *bldA* encodes the only $tRNA^{UUA}_{Leu}$ of *S. coelicolor* and is required for Red production at high phosphate concentrations.

SCB1 is one of the γ-butyrolactone autoregulators produced by *Streptomyces coelicolor* A3(2) (Takano *et al.*, 2000), which is the most genetically characterized streptomycete. This strain produces at least four antibiotics, including the blue-pigmented polyketide actinorhodin (Act) and the red-pigmented tri-pyrolle undecylprodigiosin (Red), both of which are produced in a growth phase-dependent manner (Gramajo *et al.*, 1993; Takano *et al.*, 1992; Hopwood *et al.*, 1995). The presence of SCB1 elicits the precocious production of Act and Red at the transition phase or when added exogenously to the strain. Recently, *scbR* (*Streptomyces coelicolor* A3(2) γ-

butyrolactone regulator) was cloned as the result of a collaboration between John Innes Centre, U.K., and our laboratory, and has been analyzed both in vitro and in vivo (Fig. 1.6; Takano and Bibb, personal communication; Ninagawa *et al.*, unpublished data). ScbR is a DNA-binding transcriptional regulator dependent on SCB1; it forms an autoregulatory circuit on its own gene (*scbR*) and seems to indirectly regulate the biosynthetic gene cluster of Red.

Others

These days, the biosynthetic mechanisms of many commercially useful antibiotics have been studied and the related biosynthetic gene clusters have been found and isolated. These analyses have resulted in the discovery of close homologues of the γ-butyrolactone autoregulator gene within various gene clusters. *tarA* (Engel and Scharfenstein, unpublished data), *avaR* (Atago *et al.*, unpublished data), *tylP* (Bate *et al.*, 1999), and *stbA* (Sadamoto *et al.*, unpublished data) have emerged from *Streptomyces tendae* (a fungicide nikkomycin producer), *Streptomyces avermitilis* (an antiparasitic avermectin producer), *Streptomyces fradiae* (an antibacterial agent tylosin producer), and *Streptomyces tsukubaensis* (an immunosuppresant FK506 producer), respectively. Although these predicted proteins are highly homologous with existing autoregulator receptors, especially with FarA of *S. lavendulae* FRI-5, and have a DNA-binding motif at the N-terminus portion (Fig. 1.7), their function in antibiotic production has not yet been determined.

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                                                  Helix  Turn  Helix
FarA   1:VAEQVRAIRTRQAILSAAARVFDERGYQAATISEILTVAGVTKGALYFHFQSKEDLAQGV 60
AvaR   1:MARQERAIRTRQTILVAAAEVFDEVGYEAATISDVLKRSGVTKGALYFHFTSKQELAQAV 60
StbA   1:MAQQERAIRTKLAIVTAAASVFDECGYERATIGEILARAGVTKGALYFHFPSKAALAEGV 60
TarA   1:MAQQDRAVRTRRAVLRAAAAVFAERGYAAATISEILKRAGVTKGALYFHFDSKAALAQGV 60
TylP   1:MARQERAAQTRRTIVAAAAAVFDELGYEATTIAEILKRSGVTKGALYFHFTSKEQLAQEV 60
          * * **  *      *** ** * **   **   *   ********** **  **  *
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Fig. 1.7 Amino-terminal sequences of FarA and similar proteins in the database. These proteins range from 220 (TarA) to 234 (AvaR) amino acids and the highly conserved amino-terminal sequences are shown. The helix-turn-helix motifs are indicated; the downstream "recognition"helix that binds into the major groove of the DNA taget site is especially well conserved.

In this thesis, as an important step in clarifying the IM-2-mediated regulation mechanism of secondary metabolism in *S. lavendulae* FRI-5, I investigated the function of an IM-2-specific receptor (FarA) both in vitro and in vivo. In chapter 2, I demonstrated the DNA-binding activity of FarA using gel-shift assays and localized the target sequence of this receptor by DNase I footprinting. The FarA binding sequence overlaps the –10 region and the transcriptional start site in the *farA* promoter, which was determined by high-resolution S1 nuclease mapping. These results suggest that FarA regulates its own synthesis in an IM-2-dependent manner. Before in vivo analysis, I established as described in chapter 3, a simple and reliable procedure for the conjugal transfer of DNA from *E. coli* to *S. lavendulae* using the *oriT*- and *attP*-containing plasmid pSET152. Phenotypic analysis of exconjugants revealed that integration of heterologous DNA into the ϕC31 *attB* site has no deleterious effect on IM-2-dependent gene regulation, thus providing a viable system for analyzing the regulation of secondary metabolism. In chapter 4, to confirm that FarA is actually involved in the IM-2 signaling cascade of *S. lavendulae* FRI-5 and also to establish common traits of autoregulator-dependent phenotypes, a *farA* deletion mutant of *S. lavendulae* was constructed and a phenotypic comparison between the wild-type strain and a *farA*-deleted strain was reported. Lines of evidence are present showing that FarA is involved as a negative regulator in the production of blue pigment and nucleoside antibiotics. Moreover, the FarA-IM-2 complex itself, rather than unbound FarA, has been demonstrated to be an essential component in the suppression of D-cycloserine production, showing that an autoregulator-bound receptor can have regulatory function in vivo. In chapter 5, general conclusions are provided.

Chapter 2

In vitro analysis of the butyrolactone autoregulator receptor protein (FarA) of *Streptomyces lavendulae* FRI-5 reveals that FarA acts as a DNA-binding transcriptional regulator that controls its own synthesis

2.1 Introduction.

IM-2 [(2R, 3R, 1'R)-2-(1'-hydroxybutyl-3-hydroxymethyl) γ-butanolide] of *Streptomyces lavendulae* FRI-5 is one of many γ-butyrolactone autoregulators found in streptomycetes. These autoregulators have a common 2,3-disubstitued γ-butyrolactone skeleton, and the 10 identified to date can be classified into three groups [IM-2 type (Gräfe *et al.*, 1982; Takno *et al.*, 2000), virginiae butanolide (VB) type (Gräfe *et al.*, 1983; Kondo *et al.*, 1989; Sakuda and Yamada, 1991; Yamada *et al.*, 1987) and A-factor type (Kleiner *et al.*, 1976; Mori, 1983)] based on structure at C2 side chain (Miyake *et al.*, 1989; Nihira *et al.*, 1988; Ruengjitchatchawalya *et al.*, 1995). Receptor proteins corresponding to each of the three types of autoregulators have been purified and the genes encoding them cloned and characterized (Okamoto *et al.*, 1995; Onaka *et al.*, 1995; Ruengjitchatchawalya *et al.*, 1995; Waki *et al.*, 1997). The IM-2 specific receptor protein (FarA) from *S. lavendulae* FRI-5 is a 221-amino acid protein of 24,282 Da and shows high levels of similarity to several γ-butyrolactone receptors such as A-factor receptor protein (ArpA from *S. griseus*), VB receptor protein (BarA from *S. virginiae*) and SCB1 (IM-2 type) receptor protein (ScbR from *S. coelicolor* A3(2)) (Fig. 2.1) (Takano and Bibb, personal communication). FarA is 38.5%, 48.5% and 57.1% identical to ArpA, BarA and ScbR, respectively. Although the structural differences among the three types of autoregulators are small, producing strains show a high degree of specificity toward the

corresponding autoregulator type, indicating the presence of receptor proteins of strict ligand specificity.

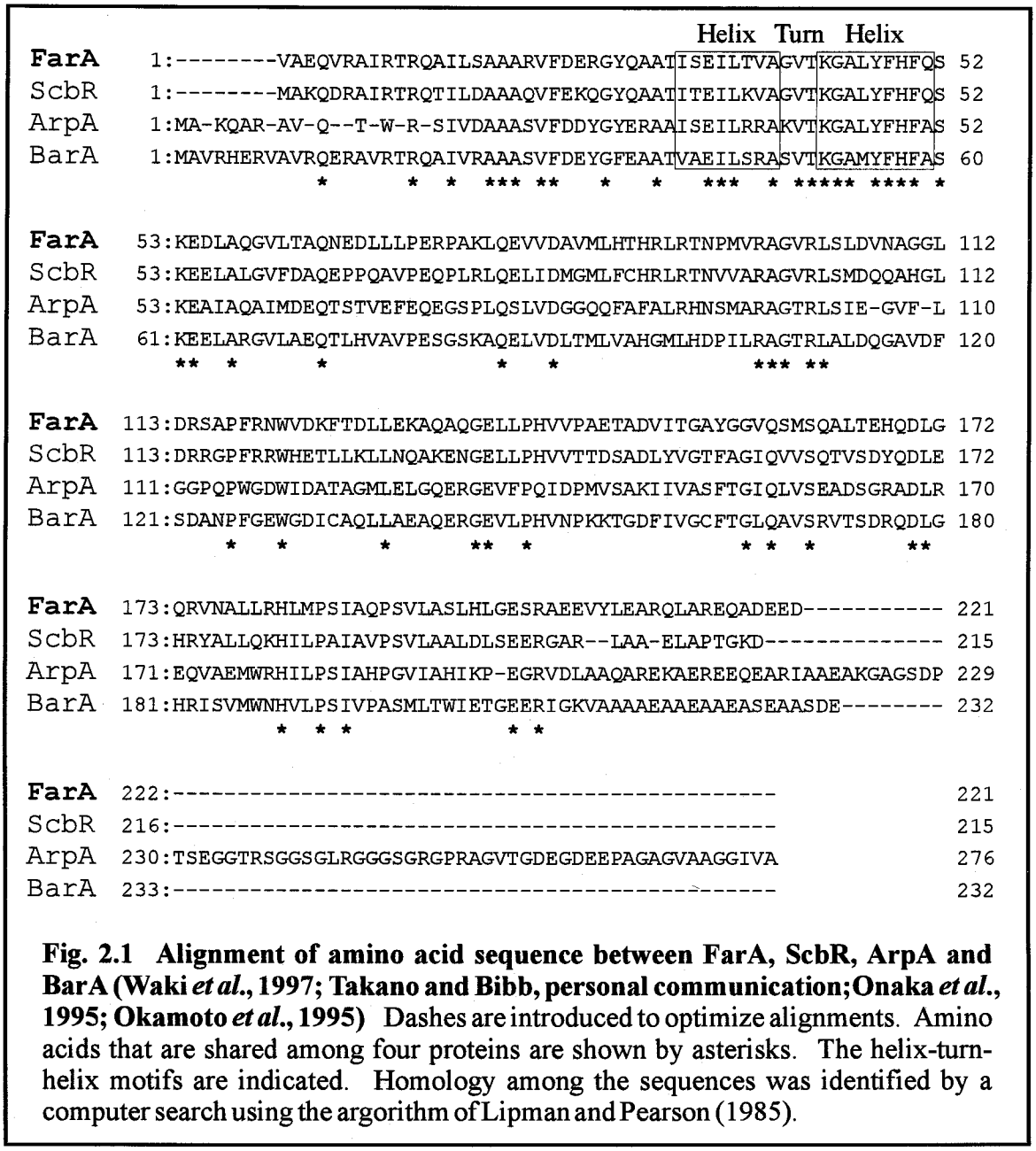

Fig. 2.1 Alignment of amino acid sequence between FarA, ScbR, ArpA and BarA (Waki *et al.*, 1997; Takano and Bibb, personal communication; Onaka *et al.*, 1995; Okamoto *et al.*, 1995) Dashes are introduced to optimize alignments. Amino acids that are shared among four proteins are shown by asterisks. The helix-turn-helix motifs are indicated. Homology among the sequences was identified by a computer search using the argorithm of Lipman and Pearson (1985).

The N-terminal region of FarA (amino acids 17–54) shows extensive homology with the other two types of autoregulator receptors. This homologous region was predicted to encode a helix-turn-helix DNA binding motif, and the DNA-binding activity is estimated to be controlled by the presence of the corresponding autoregulator (Kinoshita *et al.*, 1997; Onaka and Horinouchi, 1997). For example, in the absence of A-factor, ArpA acts as a negative regulator for both streptomycin production and sporulation by repressing

transcription of the pleiotropic regulatory gene *adpA* (Ohnishi *et al.*, 1999; Yamazaki *et al.*, 2000). In the presence of A-factor, A-factor-ArpA complex immediately dissociates from the *adpA* promoter, derepressing the transcription of *adpA* and hence triggering onset of secondary metabolism and morphological differentiation. From the presence of the conserved helix-turn-helix motif in FarA, FarA is also presumed to have DNA binding activity. However, nothing was known about the sequences recognized by FarA, or the effect of different autoregulators on the DNA binding acitivity.

In this chapter, I demonstrate that FarA has clear DNA binding activity by using gel-shift assays, and localize the FarA's target sequence by DNase I footprinting. The sequence to which FarA binds does not possess an obvious palindromic structure, but overlaps the –10 region and transcriptional start site of the *farA* promoter, which was determined by high-resolution S1 nuclease mapping. These results suggest that FarA represses its own synthesis with the aid of IM-2.

2.2 Materials and Methods.

2.2.1 Bacterial strains and plasmids.

Streptomyces sp. FRI-5 (MAFF10-06015; National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan), which was taxonomically identified as *S. lavendulae* by courtesy of Dr. S. Miyado (Meiji Seika Kaisha Ltd.), was used as a source of total RNA. The strain was grown at 28^{0}C as described previously (Hashimoto *et al.*, 1992) in a medium containing, per liter, 7.5 g of yeast extract (Difco), 7.5 g of glycerol, and 1.25 g NaCl (pH 6.5). *Escherichia coli* DH5α was used for subcloning with pUC18 as vector. DNA manipulations in *E. coli* and in *Streptomyces* were performed as described by Sambrook *et al.* (1989) and both Hopwood *et al.* (1985) and Kieser *et al.* (2000), respectively.

2.2.2 Chemicals.

All of the chemicals were of reagent or high-performance liquid chromatography (HPLC) grade, and were purchased from either Nacali Tesque, INC. (Osaka, Japan), Takara Shuzo Co., Ltd. (Shiga, Japan), or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [α-^{32}P] dCTP [3000 Ci/mmole] and [γ-^{32}P] ATP [6000 Ci/mmole] were purchased from ICN Biomedicals or Daiichi Pure Chemical Co., Ltd., Japan.

2.2.3 Preparation of radiolabeled DNA.

The following DNA fragments were used in gel-shift assays (Fig. 2.3.A): (i) the 227 bp *Sac*I-*Bgl*I fragment of pSB1 that contains the *farX-farA* intergeneric region; (ii) the 117 bp *Sac*I-*Sac*II fragment of pSS1 and (iii) the 110 bp *Sac*II-*Bgl*I fragment of pSB2 that also contains the *farX-farA* intergeneric region. These plasmids were digested with *Eco*RI

and *Hin*dIII, and the desired fragments purified by Nusieve GTG-agarose gel electrophoresis. The fragments were labeled with [α-^{32}P] dCTP. The 458 bp insert of pFP1 containing upstream sequences and part of the *farA* coding sequence (position 1341 to 1760) was amplified by PCR using ^{32}P-labeled FP1 (5'-AACTGCAGCTCATCGGCACACCACGGCC-3') and unlabeled M13 primer M3, and the PCR product used in DNase I footprinting. The probe (331 bp) for S1 nuclease mapping was generated by PCR using pFP1 as the template and ^{32}P-labeled SN1 (5'-TCCGAGATGGTGGCCGCCTGGTAGC-3') and unlabeled M13 primer RV as primers.

2.2.4 Gel–shift assays.

DNA fragments (5,000 to 10,000 cpm) that had been end-labeled with [γ-^{32}P] ATP were incubated with 23 pmol of purified rFarA in 15 µl of DNA binding buffer [20 mM triethanolamine (TEA)-HCl containing 0.1 M KCl, 20% (vol/vol) glycerol, 1 µg of poly(dI-dC):(dI-dC), pH 7.0] at 25°C for 10 min. When IM-2, VB-C or A-factor was added to the reaction mixture, the mixture was incubated at 25°C for 5 min. Protein-bound and free DNA were resolved on 5% non-denaturing polyacrylamide gels run in a high-ionic-strength buffer containing 50 mM Tris-HCl, 380 mM glycine and 2 mM EDTA, pH 8.5. The gels were dried and analyzed by autoradiography.

2.2.5 DNase I footprinting.

DNase I footprinting assays were carried out with 45 µl of the DNA-binding reaction mixture described above. After incubation at 25°C for 10min, DNase I (Boehringer Mannheim) in 100 mM $MgCl_2$-50 mM $CaCl_2$ was added to each reaction mixture to a final concentration of 40 µg, 20 µg, 10 µg and 5 µg per ml and incubated at 25°C for 1 min. The DNase I digestions were stopped by the addition of 400 µl of DNase I stop solution

(150 mM sodium acetate, 10 mM EDTA, tRNA 25 μg per ml). The reaction mixture was extracted once with phenol:chloroform (1:1,vol/vol), and the DNA precipitated with ethanol using 30 μl of Quick precip™ Plus (Edge Biosystems Inc.). The resulting pellet was resuspended in 5 μl of sequencing loading buffer (80% formamide, 10 mM EDTA, 0.1% xylen cyanol FF, 0.1% bromophenol blue). After incubation at 95°C for 3 min, 3 μl were applied to a 6% polyacrylamide-8 M Urea sequencing gel along with dideoxy DNA sequence ladders derived from primer FP1. After electrophoresis, the gels were dried and analyzed by autoradiography.

2.2.6 RNA isolation.

For RNA isolation, strain FRI-5 was grown in liquid medium (Hashimoto *et al.*, 1992) for 5 hr; IM-2 was added to a final concentration of 100 nM, and the culture incubated for a further 3 hr, which corresponded to the maximal level of production of the IM-2-induced blue pigment (Hashimoto *et al.*, 1992). RNA was extracted essentially as described (Hopwood *et al.*, 1985; Kieser *et al.*, 2000) except that the mycelium was scraped directly into modified Kirby mixture.

2.2.7 S1 nuclease mapping of the *farA* transcription start site.

High-resolution S1 nuclease mapping of the *farA* transcription start site was performed according to White, J. *et al.* (1997). RNA (40 μg) was hybridized with 30,000 cpm of probe in NaTCA buffer (Murray, 1986). After digestion with S1 nuclease (100 units) for 1 hour at 37°C, the sample was separated on a 6% polyacrylamide-8 M urea sequencing gel as described above along with DNA sequence ladders generated with the same primer.

2.3 Results and Discussion.

2.3.1 FarA binds specifically to the *farX-farA* intergeneric region.

Northern blot hybridization analysis of *farA* transcripts had shown that the addition of IM-2 during the cultivation of FRI-5 resulted in enhanced (1.5- to 1.7-fold) transcription of *farA* (Fig. 2.2; Waki *et al.*, 1997).

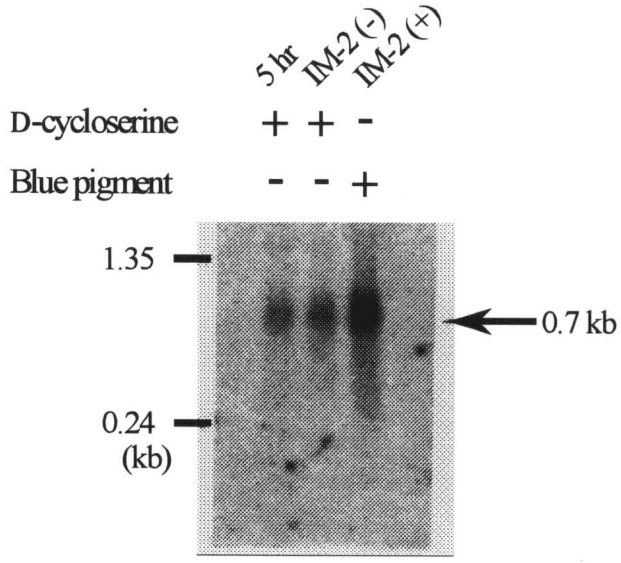

Fig. 2.2 Northern blot analysis of the *farA* transcript (Waki *et al.*, 1997). Total RNA (16 μg)from mycelia of *S. lavendulae* FRI-5 grown for 5 and 8 h (with or without IM-2 added at 5 h) was electrophoresed on a 1.0% agarose-formaldehyde gel together with size markers. Migration positions of size markers are shown at the left.

Promoter-probe analysis using the vector pIJ486 (Hopwood *et al.*, 1985; Kieser *et al.*, 2000) had identified promoter activity in the *Sac*I-*Bgl*I fragment immediately upstream of *farA* (Fig. 2.3.A; data not shown). It thus seemed possible that FarA regulated its own synthesis, and that it might therefore bind to its own promoter region. To address this possibility, gel-shift assays were performed with purified recombinant FarA (rFarA) (Waki *et al.*, 1997) and the 227 bp *Sac*I-*Bgl*I fragment (Fig. 2.3.A) containing the *farX-farA* intergeneric region as target DNA. As shown in Fig. 2.3.B (lane 4), rFarA bound to this fragment, reducing its electrophoretic mobility, while the control fragment (*rplK* from *S. virginiae*) was not retarded (Fig. 2.3.B, lane 2). To further localize the rFarA binding

sequence(s), the *Sac*I-*Bgl*I segment was separated into two fragments, namely a 117 bp *Sac*I-*Sac*II fragment and a 110 bp *Sac*II-*Bgl*I fragment that contained the *farX-farA* intergeneric region. While the *Sac*I-*Sac*II fragment showed no sign of rFarA binding (Fig. 2.3.C, lane 2), the *Sac*II-*Bgl*I fragment was retarded (Fig. 2.3.C, lane 4). Addition of the unlabeled *Sac*II-*Bgl*I fragment to a molar level equivalent to that of rFarA abolished visible retardation of the labeled fragment (Fig. 2.3.D, lanes 5 to 7). From these data, I conclude that there is a specific FarA-binding sequence present in the 110 bp *Sac*II-*Bgl*I fragment.

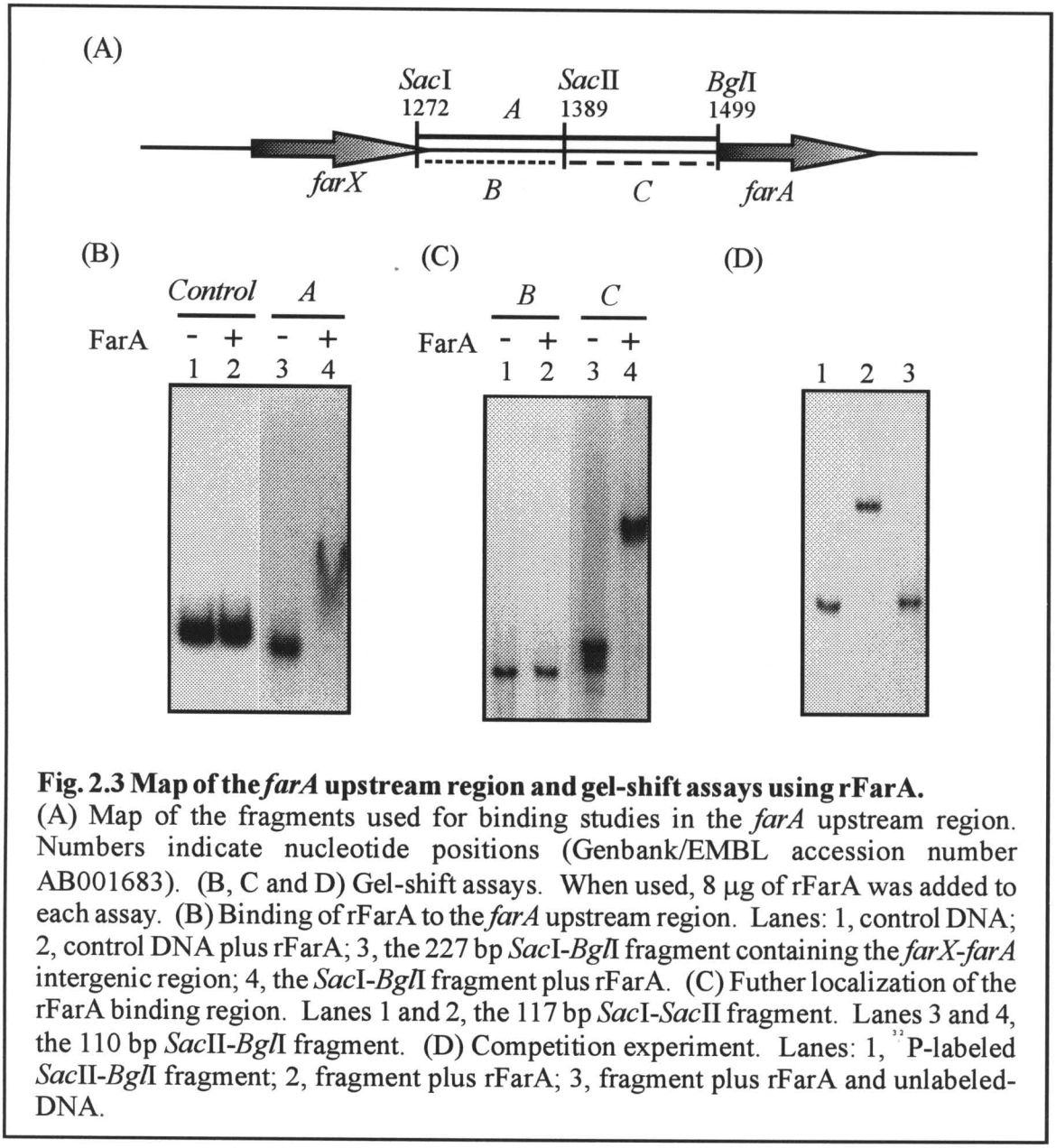

Fig. 2.3 Map of the *farA* upstream region and gel-shift assays using rFarA.
(A) Map of the fragments used for binding studies in the *farA* upstream region. Numbers indicate nucleotide positions (Genbank/EMBL accession number AB001683). (B, C and D) Gel-shift assays. When used, 8 μg of rFarA was added to each assay. (B) Binding of rFarA to the *farA* upstream region. Lanes: 1, control DNA; 2, control DNA plus rFarA; 3, the 227 bp *Sac*I-*Bgl*I fragment containing the *farX-farA* intergenic region; 4, the *Sac*I-*Bgl*I fragment plus rFarA. (C) Futher localization of the rFarA binding region. Lanes 1 and 2, the 117 bp *Sac*I-*Sac*II fragment. Lanes 3 and 4, the 110 bp *Sac*II-*Bgl*I fragment. (D) Competition experiment. Lanes: 1, ^{32}P-labeled *Sac*II-*Bgl*I fragment; 2, fragment plus rFarA; 3, fragment plus rFarA and unlabeled-DNA.

2.3.2 The DNA binding activity of FarA is controlled by IM-2.

FarA shows high ligand specificity, readily discriminating IM-2 from the structurally similar VBs and A-factor (Ruengjitchatchawalya *et al.*, 1995; Waki *et al.*, 1997). While FarA binds IM-2 with a K_d of 1.3 nM, the VBs show at least 10-fold less activity, and A-factor shows almost no affinity towards FarA. However, the ability of each ligand to influence the DNA-binding activity of FarA had not been assessed. To address this issue, the gel-shift assays were repeated using increasing concentrations of the autoregulators (Fig. 2.4).

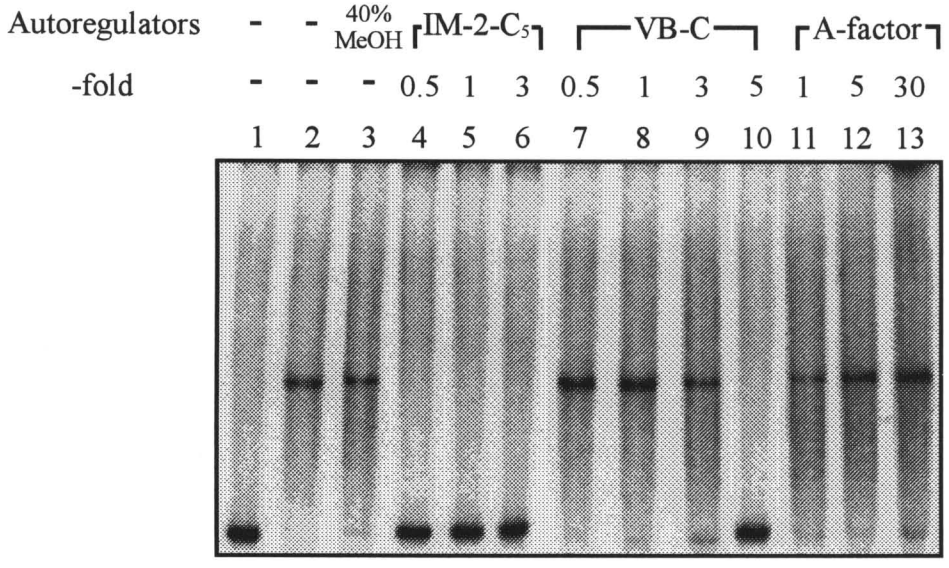

Fig. 2.4 Effect of different autoregulators on the DNA binding activity of rFarA. Lane 1, 110 bp *Sac*II-*Bgl*I fragment only; lane 2, DNA plus rFarA; lane 3, DNA plus rFarA and 40% methanol (MeOH); lanes 4 to 6, 0.5, 1 and 3 molar equivalents of IM-2; lanes 7 to 10, 0.5, 1, 3 and 5 molar equivalents of VB-C; lanes 11 to 13, 1, 5 and 30 equivalents of A-factor.

With IM-2, addition of a 0.5 mol equivalent of IM-2 with respect to rFarA completely abolished DNA binding activity (lane 4). Since B_{max} (the binding maximum) of purified rFarA for IM-2 is 0.38 mol of [^{3}H]IM-2-C$_5$/mol of rFarA monomer (Waki *et al.*, 1997), this was not surprising (the low B_{max} presumably indicates that only approximately 38% of the rFarA was capable of binding IM-2 due to inactivation during purification, and we suspect that this 38% of rFarA can bind DNA). VB-C was also able to inhibit the DNA-

binding activity of rFarA (lane 10), but was required at much higher concentrations than IM-2. The presence of a fivefold molar excess of VB-C over rFarA, 10 times of a concentration of IM-2 eliciting the same effect, completely eliminated the mobility shift. In contrast, a 30-fold molar excess of A-factor had no effect. These results agreed well with the earlier biochemical studies of the ligand specificity of rFarA (Waki *et al.*, 1997) and indicated that rFarA in its unbound form possesses DNA binding activity that is lost upon binding of IM-2. All three autoregulator receptors characterised thus far (FarA, BarA and ArpA) were found to exist as dimers under native conditions (Okamoto *et al.*, 1995; Onaka *et al.*, 1995; Waki *et al.*, 1997). Since the dimeric form of rFarA obtained by TSK G-2000SW_{XL} gel-filtration high-pressure liquid chromatography showed clear DNA binding activity (data not shown), and since rFarA remains as a dimer after binding IM-2, it seems likely that it is the dimeric form of rFarA that binds to DNA in vivo.

2.3.3 Analysis of the FarA binding site by DNase I footprinting.

To further localize the FarA binding sequence in the *Sac*II-*Bgl*I fragment, DNase I footprinting experiments were performed using the 420 bp DNA fragment (position 1341 to 1760) labeled on the coding strand by PCR amplification (Fig. 2.5). The ^{32}P-end-labeled fragment was incubated with DNase I in the presence and absence of rFarA. The region protected by rFarA contains 28 bp of the *farX-farA* intergeneric region, extending from nucleotides 1429 to 1456. This sequence, named FARE (FarA-responsive element), is located approximately 70 bp upstream of the *farA* translational initiation site and shows significant homology to one of the artificial ArpA binding sequences (Table 2.1). Onaka and Horinouchi (1997) concluded that a palindromic sequence was necessary for ArpA binding, and deduced the half site sequence 5'-GG(T/C)CGGT(A/T)(T/C)G(T/G)-3' as a consensus ArpA binding site. These sequences were obtained and recovered from a pool

of random sequence oligonucelotides by rounds of a binding/immunoprecipitation/amplification procedure with histidine-tagged ArpA and anti-ArpA antibody.

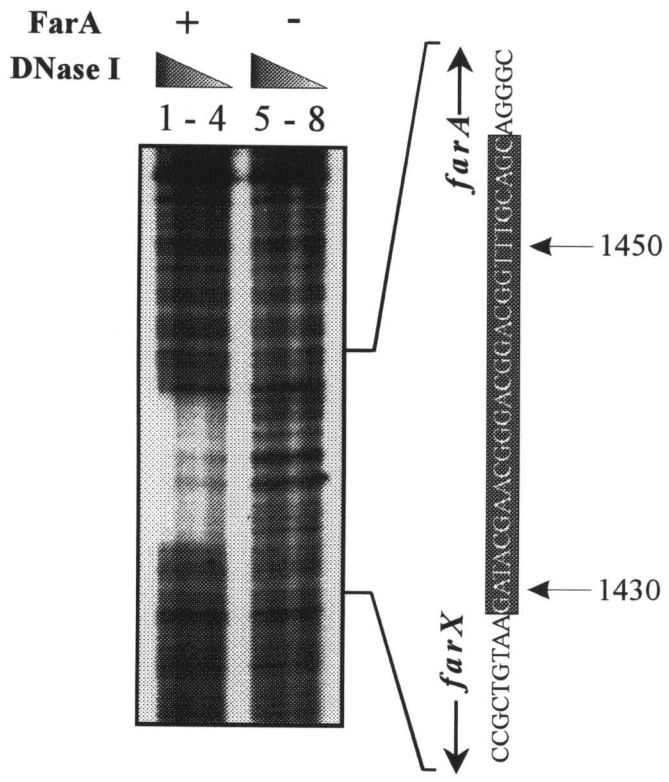

Fig. 2.5 DNase I footprinting analysis of the rFarA binding site. Lanes: 1 to 4, 14 μg of purified rFarA incubated with 40, 20, 10, and 5 μg of DNase I per ml, respectively; 5 to 8, no rFarA incubated with 40, 20, 10 and 5 μg of DNase I per ml, respectively. Protected bases are present withhin black box.

Although the homology of FARE to this ArpA consensus half-site is high (81.8% identity), FARE does not appear to form a significant palindrome. Recently, natural ArpA-binding site (ABS), which is located within promoter of gene (*adpA*), was cloned and analyzed (Ohnishi *et al.*, 1999). While ABS is highly similar to the consensus sequence of the ArpA binding site (Table 2.1), it does not seem to form a complete palindromic sequence, either. Moreover, in assuring these facts, deletion and mutation analysis of BAREs (BarA-responsive elements) (Table 2.1), to which BarA binds for transcriptional control, also indicates that the binding sequences of all autoregulator receptors are only partially

palindromic with A and T rows at both ends (Kinoshita *et al.*, 1997), suggesting that palindromic nature is not prerequisite of the binding sequences for the autoregulator receptors. The well conserved target sequences for autoregulator receptors as well as the wide distribution of autoregulators (Hashimoto *et al.*, 1992) suggests that transcriptional regulation via such binding sequences should be widespread in *Streptomyces* species.

Table 2.1 Protected region in DNase I footprint analysis and binding sites of autoregulator receptor protein from *Streptomyces* species

Region	Sequence[a]
FARE (1429-1456)	GATACGAACGGGACGGAC-GGTTTGCAGC
BARE-1[b] (BarA-responsive element)	GAGGCAAGCGAACCGCTC-GGTTTGCTGAA
BARE-2	CAAAAACAAGGCAACCGGTCTGGTTTGA
BARE-3	AGATACATACCAACCGGT-TCTTTTGA
ABS (ArpA-binding site)	AGGAACGGACCGCGC-GGTACGC
ArpA-binding consensus sequence	A C AT C GA ACCG A G CCGG T C C-GGT AT TC T G G

[a] Double underlines denote the center of the palindromic structure; arrows indicate the complementary pairs.
[b] Complementary strand.

2.3.4 Analysis of the *farA* promoter region by high-resolution S1 nuclease mapping.

The location of FARE relative to the *farA* translational initiation codon suggested that FarA might control the transcription of *farA*. To address this possibility, the transcriptional start site of *farA* was determined by high-resolution S1 nuclease protection analysis performed as described by Janssen *et al.* (1989) and White and Bibb (1997) (Fig. 2.6). A protected fragment corresponding to putative transcription start site at an A residue at position 1436, 64 bp upstream of the *farA* translational start codon, was observed. Five base pairs upstream of this putative start site lies the hexameric sequence

TAAGAT, and 16 bp upstream lies the motif TTGGCG; these sequences may act as the –10 and –35 regions, respectively, of the *farA* promoter (Fig. 2.7) (Bourn and Babb, 1995).

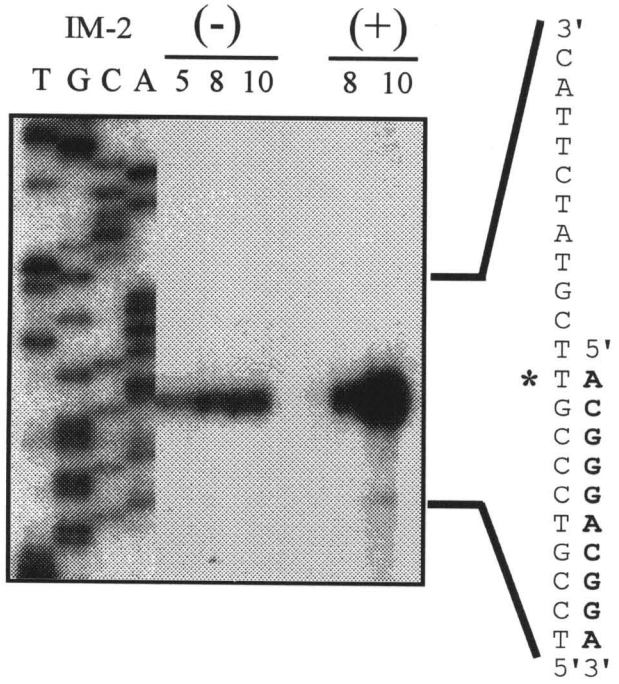

Fig. 2.6 High-resolution S1 nuclease mapping of the *farA* transcription start site and time course of *farA* expression in the presence and absence of IM-2. For RNA isolation, strain FRI-5 was grown in liquid medium (Hashimoto *et al.*, 1992) and total RNA was prepared from the mycelium of the indicated cultivation time (hours). (+), IM-2 was added to a final concentration of 100 nM at 5 h of cultivation, and cultivation continued for a further 3 or 5 h;(-), IM-2 was not added at 5 h. Lanes T, C, G and A represent sequence ladders generated with the same primer that was used to derive the end-labeled probe. Asterisk indicates the apparent transcription start site.

The binding sites of most prokaryotic transcriptional regulators (activators or repressors) either overlap the RNA polymerase binding site or are located immediately upstream of this sequence (Guilfoile *et al.*, 1992; Retzlaff and Distler, 1995). FARE covers the putative *farA* transcription initiation site and overlaps the probable –10 region of *farA*, suggesting that FarA acts as transcriptional repressor of its own synthesis by preventing RNA polymerase binding. Binding of IM-2 to FarA would then result in the dissociation of the receptor from the DNA, allowing transcription of *farA* to occur. IM-2 addition during cultivation clearly induced *farA* transcription, as detected by S1 nuclease mapping, with some basal level transcription in the absence of IM-2 (Fig. 2.6).

Fig. 2.7 Nucleotide sequence of the *farA* upstream region. The putative -35 and -10 regions of *farA* are boxed, the apparent transcription start site is denoted by an asterisk, and the putative ribosome-binding site (RBS) is underlined. The region (FARE) protected from DNase I digestion by purified rFarA is underlined with a broken line.

I propose that in the early stages of cell growth, before IM-2 production can be detected, low-level synthesis of *farA* mRNA generates sufficient FarA to repress its own transcription. During later stages of growth, when IM-2 can be detected in the culture medium, transcription of *farA* is derepressed and increases dramatically. The resultant FarA could then act to regulate the expression of other genes required for secondary metabolism.

I believe that FarA plays an important role in the regulation of secondary metabolism in *S. lavendulae*, and that it does so in conjunction with IM-2. While the production of the nucleoside antibiotics showdomycin and minimycin is induced by addition of IM-2, the production of D-cycloserine is terminated. It remains to be determined whether the biosynthetic genes for the production of these compounds are regulated by FarA, and if so, whether directly or indirectly. If they are directly controlled by FarA, many of them should be preceded by sequences similar to FARE. Our laboratory is currently in the process of isolating additional FarA binding sites that may help answering this question.

2.4 Summary.

Gel-shift assays demonstrated that FarA binds to the *farA* upstream region and that this binding is abolished in the presence of IM-2. The FarA binding sequence was localized by DNase I footprinting to a 28 bp sequence located approximately 70 bp upstream of the *farA* translational start site. High-resolution S1 nuclease mapping of *farA* transcripts revealed a putative transcription start site, located at an A residue positioned 64 bp upstream from the *farA* translation start codon and 4 bp downstream from an *Escherichia coli* σ^{70}-like –10 recognition region. The FarA binding sequence overlaps this –10 region and contains the *farA* transcription initiation site, suggesting that FarA acts as a repressor that, in the absence of IM-2, represses transcription of *farA*.

Chapter 3

Conjugal transfer of plasmid DNA

from *Escherichia coli* to *Streptomyces lavendulae* FRI-5

3. 1 Introduction.

In recent years, there has been a rapid accumulation in our understanding of the mechanisms that regulate antibiotic production and morphological differentiation in streptomycetes (Keleman and Buttner, 1998). The most significant step in acquiring this knowledge has been the establishment of mechanisms of introducing DNA into these organisms. This has permitted functional gene cloning, gene disruption and genetic complementation. There are several ways in which DNA can be introduced into streptomycetes. These include transformation with plasmid, cosmid and chromosomal DNA, transfection with DNA containing a phage replicon, phage transduction of chromosomal and plasmid DNA, conjugation between streptomycetes and intergeneric conjugation from *Escherichia coli*. Although transformation with *Streptomyces* protoplasts has been achieved as the most useful technique with a number of quite different species (Hopwood *et al.*, 1985), the procedure established for one strain is not always readily applicable to another streptomycetes. Many streptomycetes always require the strain specific conditions for protoplasts formation and regeneration, and also possesses restriction and modification system that can drastically reduce transformation frequencies, depending on the origin of the DNA. To avoid DNA degradation by restriction and modification system with transformation, several procedures have been developed such as isolating DNA from a methylation defecient *E. coli* host, first passaging DNA from *S. lividans* (MacNeil, 1988), heat treatment of protoplasts prior to transformation (Bailey and Winstanley, 1986; Engel, 1987) and in vitro modification prior

to transformation (Matsushima and Baltz, 1994), suggesting that specific procedure becomes required for different strains. However, recently, there has been a considerable increase in the use of intergeneric conjugation from *E. coli* to *Streptomyces*, which has the added benefit that it can assist in circumventing often potent restriction barriers (MacNeil, 1988).

Intergeneric transfer of plasmids from *E. coli* to *Streptomyces* was demonstrated after modifying the mobilization system established by Simon *et al.* (1983) for Gram-negative bacteria. The vectors, used in conjugation, contain *oriT* from the IncP-group plasmid RP4 (also designated RP1/RK2), and require transfer functions to be supplied *in trans* by the *E. coli* donor strain. The representative of *E. coli* strain in conjugation is generally either the methylation proficient S17-1 (Simon *et al.*, 1983), which carriers an integrated derivative of RP4, or the methylation deficient ET12567 (MacNeil *et al.*, 1992) carrying the nontransmissible pUZ8002 (Paget *et al.*, 1999). There are several advantages to using conjugation from *E. coli* as a means of introducing DNA into *Streptomyces*. First, it is simple and does not rely on the development of procedures for protoplast formation and regeneration. Next, restriction barriers may be by-passed or severely reduced by the transfer of single-stranded concatemers of plasmid DNA (Matsushima *et al.*, 1994). Finally, a variety of versatile *oriT* vectors are available that permit site-specific or insert-directed chromosomal integration, and the production of required constructs is considerably facilitated since these vectors replicate in *E. coli*.

Streptomyces lavendulae FRI-5 produces IM-2, an extracellular regulatory factor (Sato *et al.*, 1989), which belongs to the family of γ-butyrolactone autoregulators and plays an important role on regulating production of secondary metabolites. In chapter 2, the IM-2 specific receptor protein (FarA) has been already characterized in vitro, indicating that FarA is likely to act as a transcriptional repressor in the signal transduction

pathway that triggers secondary metabolism (Kitani *et al.*, 1999). However, understanding the in vivo function of FarA has been hindered by the lack of an effective means to introduce DNA into the strain.

In this chapter, I established a simple and reliable procedure for the conjugal transfer of DNA from *E. coli* to *S. lavendulae* using the *oriT*- and *attP*-containing plasmid pSET152. Phenotypic analysis of exconjugants revealed that integration of heterologous DNA into the phage ϕC31 *attB* site has no deleterious effect on IM-2-dependent gene regulation, thus providing a viable system for analyzing the regulation of secondary metabolism in this organism.

3.2 Materials and Methods.

3.2.1 Bacterial strains, plasmids and bacteriophages.

Streptomyces lavendulae FRI-5 (MAFF10-06015; National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan) was used as a recipient throughout this study. Spores were obtained after 7 days growth at 28°C on ISP medium 2 (Difco) (ISP2), and stored as 20% glycerol suspensions at –80°C. Bacteriophages KC212 and KC213 (Bruton and Chater, personal communication), carrying an apramycin resistance gene (*apr*) and *attP*, were used in lysogenization experiments. The methylation-deficient *Escherichia coli* strain ET12567 (*dam-13*::Tn9, *dcm-6*, *hsdM*, *hsdS*) (MacNeil *et al.*, 1992) containing pUZ8002 was used as donor in intergeneric conjugations. pUZ8002 (Paget *et al.*, 1999) is a RK2 derivative with a defective *oriT* (*aph*); it is not self-transmissible but supplies mobilization functions for *oriT*-containing plasmids *in trans*. The *E. coli–Streptomyces* shuttle vectors pSET152 (Bierman *et al.*, 1992) and pPM925 (Servant *et al.*, 1993) possess ϕC31 *attP* and *int* (Lomovskaya *et al.*, 1980), which direct site-specific integration of the plasmids into the streptomycetes chromosomal *attB* site. Procedures for standard DNA manipulations in *E. coli* and *Streptomyces* were as described in Sambrook *et al.* (1989) and both Hopwood *et al.* (1985) and Kieser *et al.* (2000), respectively.

3.2.2 Intergeneric conjugation from *E. coli* to *S. lavendulae* FRI-5.

The donor *E. coli* ET12567 (pUZ8002) harboring pSET152 or pPM925 was grown in L-broth at 37°C with reciprocating shaking (120 strokes per min) to an OD_{600} of about 0.4, the cells were collected by centrifugation, washed twice with L-broth, collected again and finally suspended in 0.1 volume of fresh L-broth. Unless otherwise stated, *S. lavendulae*

FRI-5 spores (10^8 to 10^{10} spores per conjugation) were incubated in 2 x YT for 10 min at 40°C. The spores, with or without heat treatment, were mixed with the *E. coli* donor cells in a 1:1 volume ratio, and the mixture was spread on MS agar (mannitol plus soya flour) (Hobbs *et al.*, 1989) plus 10 mM $MgCl_2$, ISP2 plus 10 mM $MgCl_2$ or R5 agar (Hopwood *et al.*, 1985; Kieser *et al.*, 2000), respectively. After incubation at 30°C for 18 h, the plates were overlaid with 1 ml of water containing 500 μg nalidixic acid (kill to *E. coli*) and 1 mg apramycin (for pSET152) or streptomycin (for pPM925), and incubated at 28°C for a further 5 - 7 days, until exconjugants appeared.

3.2.3 Southern blot hybridization.

DNA samples were separated on 1% agarose gels, and transferred to Hybond-N+ (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. Hybridizations were carried out at 65°C for 2 h in rapid hybridization buffer (Amersham Pharmacia Biotech). Probe A, containing the *apr* gene (Fig. 3.4), was generated by PCR from pSET152 using aprN-2 (5'-CCCCGGCGGTGTGCTG-3') and aprC-1 (5'-CATGGATCCGACGTCGCGGTGAGTTCAGGC-3') as primers. The *farA* probe was PCR-amplified from pMW101, a pUC19 containing an 8 kb *Pst*I fragment (Waki *et al.*, 1997) with farAN-3 (5'-CGGGATCCTCATCGGCACACCACGGCCCG-3') and farAC-3 (5'-CGGGATCCTGCACAGGGGAAAGCGGA-3') as primers. These probes were labeled with [α-^{32}P] dCTP and by random oligonucleotide priming.

3.2.4 Phenotypic analyses of exconjugants.

IM-2 binding activity and induction of blue pigment production were determined as described previously in (Ruengjitchatchawalya *et al.*, 1995) and (Hashimoto *et al.*, 1992), respectively. Plate assays with *Bacillus subtilis* PCI219 were used to monitor antibiotic

production (Nihira *et al.*, 1988). To assess morphological differentiation, spores of the wild-type strain and exconjugants were streaked on plates of ISP2, oatmeal agar (Hashimoto *et al.*, 1992), MS agar, MM agar plus mannitol, R2 agar (Hopwood *et al.*, 1985; Kieser *et al.*, 2000) and modified SMMS agar (Takano *et al.*, 2000).

3.3 Results and Discussion.

3.3.1 Effect of heat treatment on the viability of *S. lavendulae* FRI-5 spores.

To effect intergeneric plasmid transfer from *E. coli* to streptomycetes, recipient spores are often subjected to heat treatment (for instance, 50°C for 10 min for *Streptomyces coelicolor* A3(2) (Hopwood *et al.*, 1985; Kieser *et al.*, 2000)) before being mixed with *E. coli* donor cells; this is because spore germination was thought to be necessary for efficient conjugation (Flett *et al.*, 1997). Thus the viability of *S. lavendulae* FRI-5 spores after different heat treatments was first assessed. While spores incubated at 50°C quickly lost viability (Fig. 3.1.A), incubation at 40°C or lower for 10 min had little effect (Fig. 3.1.B). Phase contrast microscopy confirmed that incubation at 40°C for 10 min induced spore germination. Consequently, a 10 min incubation at 40°C was used in all subsequent conjugation experiments.

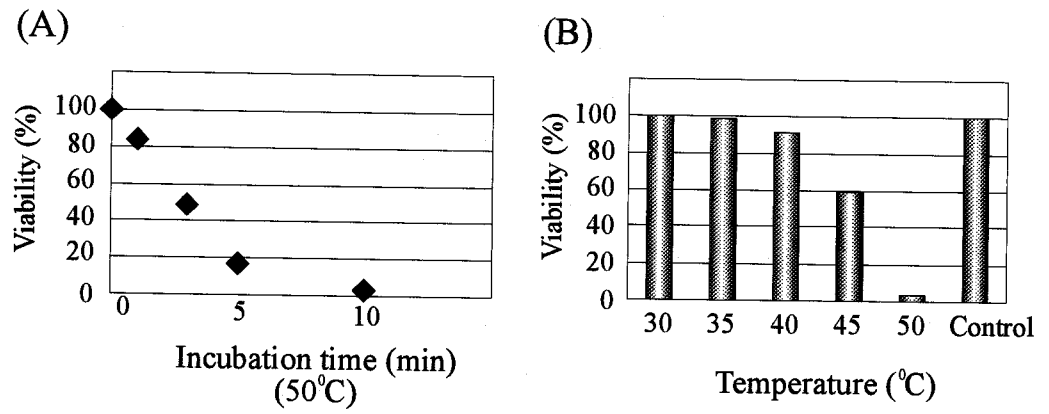

Fig. 3.1 Effect of (A) incubation time and (B) temperature on the viability of *S. lavendulae* FRI-5 spores. (A) A spore suspension of *S. lavendulae* FRI-5 was diluted to 10^3 spores per ml, and incubated at 50°C for the time indicated. (B) Spores (10^3 per ml) in 2 x YT medium were incubated for 10 min at the temperature indicated. Viability was determined by counting colonies on ISP2 medium after incubation at 28°C for 7 days. The data are the average of two independent experiments, and expressed as a percent of the colony count obtained in the absence of heat treatment.

3.3.2 Intergeneric conjugation.

Before attempting intergeneric conjugation, the presence of a suitable ϕC31 *attB* site(s) in the *S. lavendulae* FRI-5 genome was assessed by infecting the strain with the ϕC31 derivatives KC212 and KC213 (Fig. 3.2.A). These phages contain the ϕC31 *attP* and *int* functions, and the *apr* gene, which confers resistance to apramycin. Both phages yielded apramycin resistant lysogens (Fig. 3.2.B), indicating that *S. lavendulae* possessed a ϕC31 *attB* site(s) that should also be available for site-specific integration by suitable conjugative plasmids.

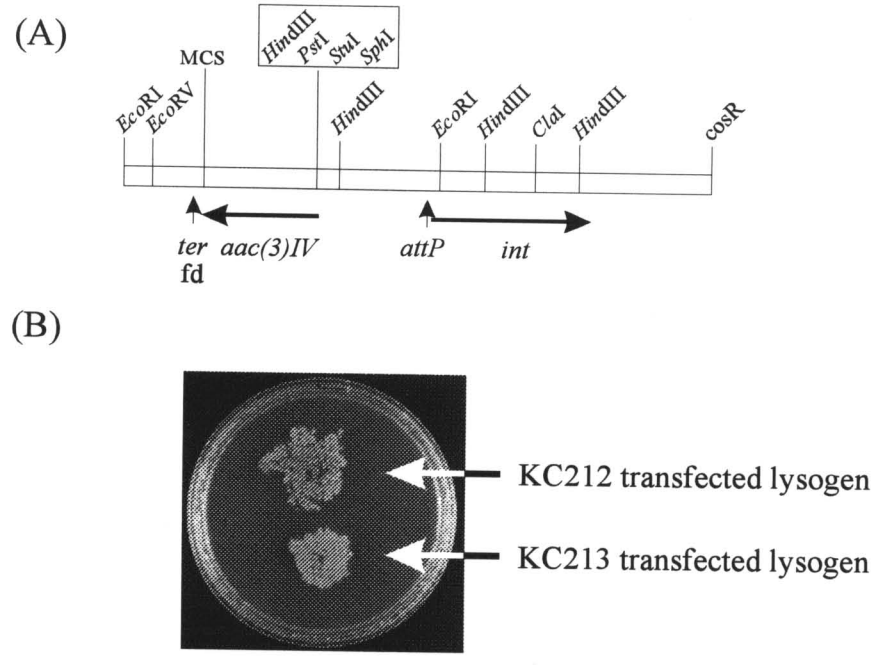

Fig. 3.2 Restriction endonuclease map of the ϕC31 derivative phage (KC212) and transfection of *S. lavendulae* FRI-5. (A) Restiriction endonuclease map in the right arm of the ϕC31 derivative phage (KC212, 70 kb). Left *Pst*I end of KC211 joined by *Stu*I site-containing linker to rightmost *Sph*I end of ϕC31. Unique sites of this phage are *Bgl*II, *Bam*HI, *Pst*I and *Stu*I. Abbreviations; MCS, Multi cloning sites; *ter*, terminator; *aac(3)IV*, apramycin resistance gene; *attP*, attachment site; *int*, integrase. (B) transfection with KC212 and other derivative phage (KC213, 71 kb). Lysogens were selected with R5 medium containing 20 μg of apramycin per ml.

For conjugal transfer from *E. coli* (Molle *et al.*, 2000), we selected *E. coli* ET12567 harboring pUZ8002 as donor, and pSET152 and pPM925 as the conjugative plasmids (Fig. 3.3). Since neither plasmid possesses an origin of replication that is functional in

streptomycetes, but since both plasmids contain *oriT* and the ϕC31 *attP* and *int* genes, they can only exist in *S. lavendulae* FRI-5 as chromosomally integrated forms.

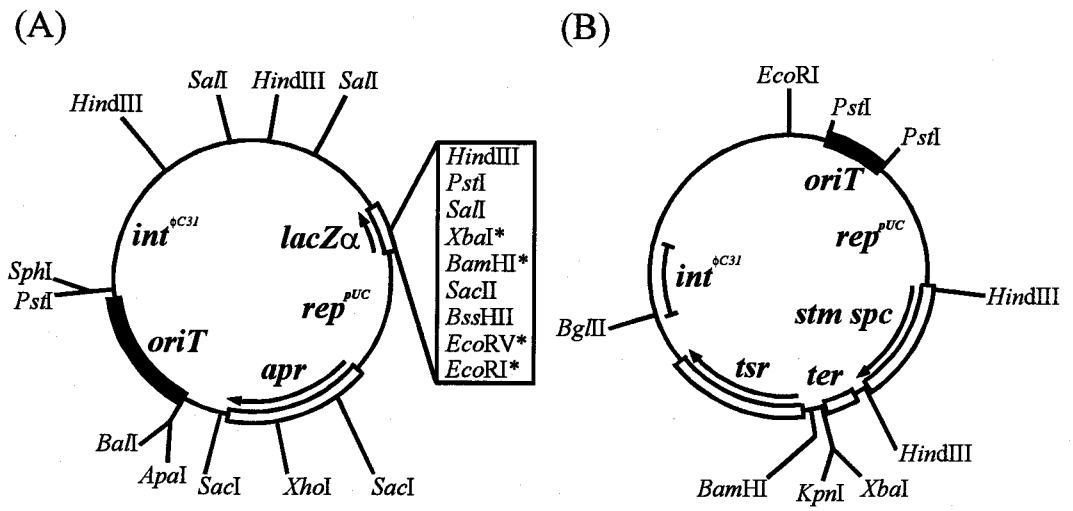

Fig. 3.3 Restiriction endonuclease maps of two plasmids for conjugation. Both plasmids can replicate in *E. coli*. (A) pSET152 (5.5 kb) (Bierman *et al.*, 1992) is *oriT* RK2 plasmids containing *aac(3)IV*, the apramycin resistance gene. (B) pPM925 (11.4 kb) (Servant *et al.*, 1993) has sepctinomycin and streptomycin resistance gene (*aadA*) and thiostrepton resistance gene (*tsr*).

Spores of *S. lavendulae* FRI-5, with or without heat-treatment, were mated with *E. coli* ET12567 (pUZ8002) harboring either pSET152 or pPM925. While pSET152 gave large numbers of exconjugants (Table 3.1), pPM925 gave none under any conditions. This may reflect the larger size of pPM925 (11.4 kb compared to the 5.5 kb of pSET152, see Fig. 3.3). An effect of plasmid size on the efficiency of intergeneric conjugation was also reported by Flett *et al.* (1997) for *S. coelicolor* A3(2). The efficiency of pSET152 transfer varied markedly with the medium used: MS agar containing 10 mM $MgCl_2$ consistently gave frequencies of about 10^{-6} exconjugants per recipient regardless of the recipient/donor ratio. ISP2 containing 10 mM $MgCl_2$ gave 1.6 x 10^{-5} to 3.9 x 10^{-8} exconjugants per recipient, with higher conjugation frequencies at lower recipient/donor ratios. R5 agar gave no transconjugants. Unlike *S. coelicolor* A3(2) (Flett *et al.*, 1997) or *Streptomyces toyocaensis* (Matsushima and Baltz, 1996), germination of recipient spores prior to mixing with the *E. coli* donor did not increase the frequency of

conjugation.

Table 3.1 Conjugal transfer of pSET152 from *E. coli* ET12567 (pUZ8002) to *S. lavendulae* FRI-5

Heat shock[a]	Number of recipient spores	Exconjugants per recipient[b]		
		MS + $MgCl_2$	ISP2 + $MgCl_2$	R5[c]
−	10^{10}	9.7×10^{-6}	7.0×10^{-8}	−
	10^{9}	6.3×10^{-6}	5.4×10^{-7}	−
	10^{8}	3.8×10^{-6}	**1.6×10^{-5}**	−
+	10^{10}	8.8×10^{-6}	3.9×10^{-8}	−
	10^{9}	4.5×10^{-6}	4.6×10^{-7}	−
	10^{8}	2.4×10^{-6}	1.4×10^{-5}	−

[a] Conditions for heat shock were 40°C 10 min.
[b] Values represent average frequecies from two independent experiments.
[c] - indicates no exconjugants.

3.3.3 Integration of pSET152 occurs at a unique ϕC31 *attB* site.

To confirm chromosomal integration of pSET152, exconjugants were analyzed by Southern blot hybridization (Fig. 3.4). Using ^{32}P-labeled pSET152 as a probe, two *Bam*HI fragments of about 15 kb and 3.6 kb were detected in all of the exconjugants tested (Fig. 3.4.A), while probe A containing the *apr* gene hybridized only to the upper 15 kb *Bam*HI fragment (Fig. 3.4.B), and to two *Sac*I fragments of about 2.4 kb and 1 kb (Fig. 3.4.C). All of the exconjugants showed identical hybridization patterns, suggesting that *S. lavendulae* FRI-5 contains a unique ϕC31 *attB* site, and that pSET152 integrates into the chromosome as shown in Fig. 3.4.D.

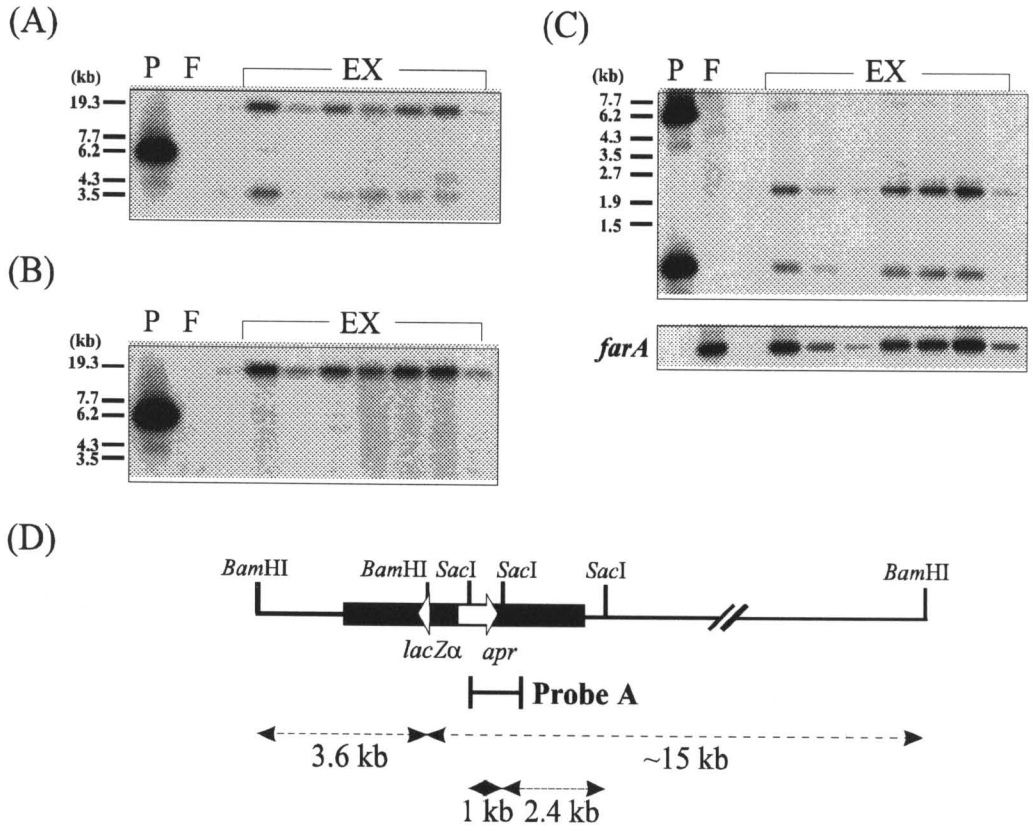

Fig. 3. 4 Southern blot analysis of exconjugants containing pSET152. P, F and EX indicate pSET152, genomic DNAs from the wild-type strain, and from seven independent exconjugants, respectively. pSET152 (5.5 kb) and genomic DNA samples digested with *Bam*HI (A and B) or *Sac*I (C) were hybridized with ^{32}P-labeled pSET152 (A) or probe A containing the *apr* gene (B and C), respectively. Size markers (kb) are indicated on the left of the gels. *farA* indicates a 2.1 kb *Sac*I fragment detected with a ^{32}P-labeled probe containing *farA* on the same membrane, and serves as a hybridization control. (D) Restriction endonuclease map of the chromosomal *attB* site containing the integrated pSET152. Arrows indicate the coding regions of *lacZα* and *apr*. The boxed region represents pSET152.

3.3.4 Plasmid integration has no effect on phenotype.

The presence of heterologous DNA in streptomycetes, either chromosomally integrated or plasmid-borne, has sometimes resulted in a reduction in antibiotic productivity and/or a reduction in growth (Voeykova *et al.*, 1998). However, the *S. lavendulae* FRI-5 exconjugants containing pSET152 grew at the same rate as the wild-type strain in liquid culture and produced similar levels of antibiotic. They were also indistinguishable from the wild-type strain in their morphological characteristics on a

range of different solid media (Fig. 3.5; MS agar and R2 medium, data not shown).

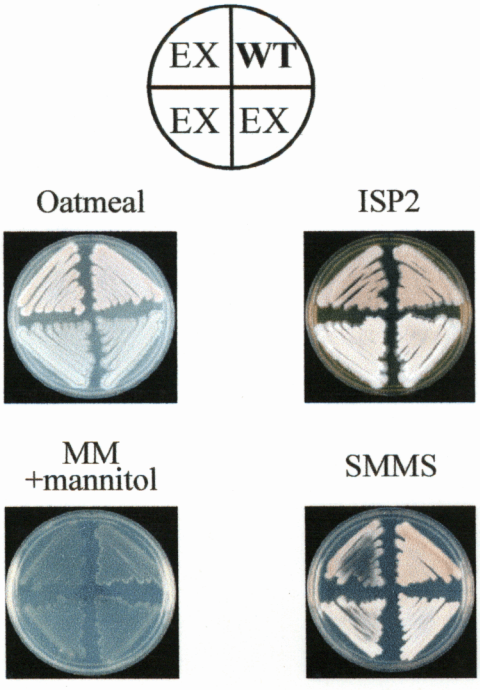

Fig. 3.5 Morphology on several kinds of medium. Abbreviations; WT, spores of wild-type; Ex, spores of exconjugant containing pSET152 on chromosome. Medium used were prepared as described in Materials and Methods. These plates were incubated for 7 days at 28°C.

Thus integration of DNA at the ϕC31 *attB* site appears to be neutral with regard to both morphological differentiation and antibiotic production. To further assess whether plasmid integration might have influenced the IM-2 signal transduction cascade, the ability of IM-2 to induce the production of blue pigment and the binding activity of the IM-2 receptor protein FarA was determined in exconjugants and their parental strain (Hashimoto *et al.*, 1992; Ruengjitchatchawalya *et al.*, 1995). All of the exconjugants showed induction of blue pigment production 2 h after the addition of IM-2 to a 5-h liquid culture, as in the parental strain. Moreover, the IM-2 binding activity in crude cell-extracts of both strains was essentially identical (Table 3.2).

Table 3.2 IM-2 binding activity of wild-type *S. lavendulae* FRI-5 and exconjugants containing pSET152.

	IM-2 binding activity (pmol/mg-protein)	Percentage of control
Wild-type	2.21 ± 0.28	100 ± 13
Exconjugants	2.08 ± 0.09	94 ± 4

Wild-type strain and three independent exconjugants were cultivated in medium B (Hashimoto *et al.*, 1992), and IM-2 binding activity in the mycelia was measured as described in Materials and Methods. Data are expressed as an average of triplicate assays with standard deviation.

These results indicated that plasmid integration with chromosomal DNA of *S. lavendulae* FRI-5 has no detrimental effect on phenotype.

Importantly, this procedure will allow targeted gene disruption and mutant complementation in *S. lavendulae* FRI-5, which enable to investigate the complex regulatory cascade of IM-2-dependent regulation on secondary metabolism in this strain. Indeed, in the next chapter, disruption of an autoregulator receptor gene (*farA*) by the established conjugation system gave clues for understanding the regulation.

3.4 Summary

I have established a reliable system for the intergeneric transfer of DNA from *E. coli* ET12567 (pUZ8002) to *S. lavendulae* FRI-5, and have determined the optimal conditions using pSET152 on two kinds of media: MS agar medium containing 10 mM $MgCl_2$ gave frequencies of 2.4 x 10^{-6} exconjugants per recipient; ISP2 medium containing 10 mM $MgCl_2$ gave 1.6 x 10^{-5} exconjugants per recipient; R5 medium gave no transconjugants. These results revealed that frequencies of conjugation in this strain depend on kinds of media and the length of DNA to be introduced. Germination of recipient spores prior to mixing with the *E. coli* donor did not increase the frequency of conjugation. I also confirmed that chromosomal integration occurs at a unique ϕC31 *attB* site and does not influence morphological differentiation or IM-2-dependent signal transduction.

Chapter 4

Gene replacement analysis of the butyrolactone autoregulator receptor (FarA) reveals that FarA acts as a dual-function regulator in secondary metabolism of *Streptomyces lavendulae* FRI-5

4.1 Introduction

Among factors known to affect antibiotic production and/or morphological differentiation in streptomycetes, γ-butyrolactone autoregulators have been shown in several streptomycetes to serve as extracellular signaling molecules determining the onset of the two noteworthy characteristics. Among the known γ-butyrolactone autoregulators, the earliest known is A-factor (Hara and Beppu, 1982; Khoklov, 1988; Khoklov *et al.*, 1967), which is required for both streptomycin production and sporulation in a streptomycin$^-$(str$^-$) - sporulation$^-$(spo$^-$) mutant of *S. griseus*. Other well-studied γ-butyrolactone autoregulators are virginiae butanolides (VBs) which control the virginiamycin production in *S. virginiae* (Kondo *et al.*, 1989; Yamada *et al.*, 1987), and SCB1 which induces the precocious production of actinorhodin and undecylprodigiosin in *S. coelicolor* A3(2) (Takano *et al.*, 2000). The most unique among the γ-butyrolactone autoregulators is IM-2 of *S. lavendulae* FRI-5. In contrast to the solely positive effects exerted by other members of the autoregulator group, IM-2 is capable of not only switching on productions of a blue pigment and the nucleoside antibiotics showdomycin (SHM) and minimycin, but also switching off production of the antituberculosis antibiotic D-cycloserine (DCS) (Hashimoto *et al.*, 1992).

In vitro studies of an IM-2 specific receptor protein (FarA) (Ruengjitchatchawalya *et al.*, 1995; Waki *et al.*, 1997; Kitani *et al.*, 1999) have indicated that FarA is a dimeric

DNA binding protein that, in the absence of IM-2, recognizes and binds to specific DNA sequences situated in the promoter region of a target gene. IM-2 binding to FarA causes FarA to dissociate from the DNA, which in turn allows the transcription of the target gene to occur. Similar data have been obtained in vitro for a VB specific receptor (BarA) (Kinoshita *et al.*, 1997; 1999) and an A-factor specific receptor (ArpA) (Onaka and Horinouchi, 1997; Ohnishi *et al.*, 1999), suggesting that all the autoregulators express common activity as transcriptional repressors. Yet, a common in-vivo trait on the autoregulator-dependent cascade has not been drawn from phenotypic analyses of receptor deficient mutants. When an ArpA$^-$ mutant of *S. griseus* was created in the A-factor minus background, the defect in sporulation was restored with even earlier initiation than the wild-type (str$^+$ spo$^+$) strain, and the defect in streptomycin production was restored to further 10-fold overproduction to that of the wild-type strain (Miyake *et al.*, 1990), indicating that ArpA solely acts as a repressor of the two processes. However, no morphological difference was observed between the wild-type *S. virginiae* and a *barA*-null mutant (Nakano *et al.*, 1998; 2000), indicating that VB specific receptor BarA is not involved in morphological differentiation. Furthermore, in the *barA*-null mutant, virginiamycin production was suppressed to only 10% of that of the wild-type strain and biosynthesis of VB itself was abolished, the latter trait of which is not clear for ArpA, because the ArpA deficient mutant was created in the A-factor minus background. Therefore, it is apparent that further investigation of another autoregulator-dependent cascade needed in order to deteemine a common trait that will enable us to predict or manipulate the autoregulator-dependent secondary metabolism in *Streptomyces* species.

In this chapter, to confirm that FarA is actually involved in the IM-2 signaling cascade of *S. lavendulae* FRI-5 and also to identify common traits of autoregulator-dependent phenotypes, a *farA* deletion mutant of *S. lavendulae* was constructed using

homologous recombination, and phenotypic comparison between the wild-type strain and a *farA*-deleted strain was reported. Similar to the case of VB biosynthesis in *S. virginiae*, FarA was found to be regulating the IM-2 biosynthesis in *S. lavendulae*. Lines of evidence are presented showing that FarA is involved as a negative regulator in the production of the blue pigment and the nucleoside antibiotics. Moreover, FarA-IM-2 complex itself, rather than the unbound FarA, was demonstrated to be an essential component in the suppression of D-cycloserine production, showing for the first time that an autoregulator-bound receptor can express a regulatory function in vivo.

4.2. Materials and Methods

4.2.1 Bacterial strains, plasmids, growth conditions, and conjugal transfer of DNA from *E. coli* to *S. lavendulae* FRI-5.

Streptomyces lavendulae FRI-5 (MAFF10-06015; National Food Research Institute, Ministry of Agriculture, Forestry and Fishers, Tsukuba, Japan) was cultured as described previously (Hashimoto *et al.*, 1992) at 28°C in medium B (containing [grams per liter] yeast extract, 7.5; glycerol, 7.5; NaCl, 1.25 (pH 6.5) for antibiotic production, in liquid medium containing each half volume of YEME (Hopwood *et al.*, 1985; Kieser *et al.*, 2000) and TSB (Oxoid) for preparation of total DNA and on ISP medium 2 (Difco) for spore formation. For conjugal transfer of DNA into *S. lavendulae* FRI-5, the methylation-deficient *Escherichia coli* strain ET12567 (*dam-13*::Tn9, *dcm-6*, *hsdM*, *hsdS*) (MacNeil *et al.*, 1992) containing the RP4 derivative pUZ8002 (Paget *et al.*, 1999) was used as the donor. *E. coli* K-12 strain JM101 (Toyobo) was used for routine subcloning. The plasmids used were pIJ8606 (Sun and Bibb, personal communication), a pIJ2925 (Janssen and Bibb, 1993) derivative containing a thiostrepton resistance gene (*tsr*), pKC1132 (Bierman *et al.*, 1992), and pSET152 (Bierman *et al.*, 1992). Procedures for standard DNA manipulation in *E. coli* and *Streptomyces* were described in Sambrook *et al.* (1989) and both Hopwood *et al.* (1985) and Kieser *et al.* (2000), respectively. All chemicals were of reagent or HPLC grade and were purchased from Nacalai tesque, Takara Shuzo, or Wako Pure Chemical Industries.

4.2.2 Construction of a *farA* deletion mutant and a *farA* complemented strain.

A 2.8 kb-*Eco*RI (blunt-ended)-*Bst*PI fragment containing 5'-upstream plus 5'-123 bp of *farA* was isolated from pMW101, a pUC19 derivative containing an 8 kb-*Pst*I fragment

(Waki *et al.*, 1997), and the fragment was cloned into *Eco*RI-*Sma*I sites of pIJ8606 to give pSG101. A 2.8 kb-*Pvu*II-*Pst*I fragment carrying 3'-30 bp plus 3'-downstream of *farA* from pMW101 was cloned into *Pst*I and blunt-ended *Sph*I sites of pSG101, generating pSG102. This reconstructed a contiguous 5.6 kb segment of the chromosome except that a 510 bp-*Bst*PI-*Pvu*II fragment internal to *farA* was replaced with the 1.1 kb-*tsr* gene. The entire 6.7 kb-insert was recovered from pSG102 as an *Eco*RI-*Pst*I fragment and cloned into *Eco*RI-*Pst*I sites of pKC1132, yielding pSG103.

E. coli ET12567 (pUZ8002) transformed with pSG103 was conjugated with *S. lavendulae* FRI-5 K101 as previously described (Kitani *et al.*, 2000). Exconjugants in which the plasmid pSG103 had presumptively integrated at the *farA* locus by a single crossover via homologous recombination were selected with apramycin. After three rounds of incubation at 28^{0}C on ISP medium 2 containing 5 μg of thiostrepton per ml, putative *farA*::*tsr* deletion mutants formed from the second crossover were detected by their apramycin sensitivity. One of the strains was designated strain K104. To complement the *farA* deletion mutant (K104), a 2.1 kb-*Sac*I fragment containing the entire *farA* with promoter was isolated from pMW101 and cloned into *Sac*I-digested and blunt-ended pSET152, generating pSG104. After conjugal transfer of pSG104 from *E. coli* ET12567 (puZ8002) to strain K104, apramycin-resistance exconjugants were obtained and designated strain K105. The resulting strains were analyzed by Southern hybridization. The probe used was a 902 bp-*farA*-internal fragment amplified by PCR from pMW101 using farAN-3 (5'-CGGGATCCTCATCGGCACACCACGGCCCG-3') and farAC-3 (5'-CGGGATCCTGCACAGGGGAAAGCGGA-3') as primers.

4.2.3 IM-2 binding assay.

Crude extracts for IM-2 binding assay were prepared as described in

Ruengjitchatchawalya *et al.* (1995). IM-2 binding activity was routinely assayed using the ammonium sulfate precipitation method (Kim *et al.*, 1990) with [^{3}H]IM-2-C_5 (10 pmol, 40 Ci/mmol) in the presence and absence of non-labeled IM-2-C_5 (15 nmol, 1,500-fold molar excess). The radioactivity in the solution was measured with a liquid scintillation counter (model LS6000; Beckman).

4.2.4 Determination of blue pigment (BP).

At the indicated times, supernatants were obtained by centrifugation (15,000 x g, 4^{0}C, 10 min) of culture broths in medium B, filtrated through 0.2-μm filters, and absorbance at 590 nm was measured.

4.2.5 Analysis of showdomycin (SHM) and D-cycloserine (DCS) production.

For SHM production, culture broth (60 ml, medium B) in a 500 ml-baffled flask was collected at 31-h cultivation, mycelia was removed by suction filtration, and the filtrate adjusted to pH 7.0. The filtrate was applied to an active charcoal column (5g) followed by washing with 100 ml of water, and the absorbed compounds were eluted with 200 ml of methanol. The methanol eluent was evaporated, dissolved in 5 ml of water, lyophilized and redissolved in 1 ml of water for bioassay. Bioassay of SHM was performed by measuring clear-zone formation with *Bacillus subtilis* PCI219 as a test organism on glucose-Simmone's agar medium as described by Nishimura *et al.* (1964) after incubation for 2 to 3 days at 30^{0}C. Authentic SHM (a generous gift from Shionogi Pharmaceuticals) was used as standard.

For DCS production, samples in medium B were withdrawn at the indicated times and clarified by filtration through 0.2-μm filters. Aliquots (50-μl) were separated and quantified by high-pressure liquid chromatography (HPLC) (cation-exchange column;

Senshu pak SCX-1251-N, Senshu scientific Co., Ltd.) with 10 mM ammonium acetate (pH 5.0) as solvent and detection at 210 nm using authentic DCS (Sigma) as the standard.

4.2.6 Morphological assessment.

Spores of the wild-type strain, a *farA* deletion mutant (strain K104), and a *farA* complemented strain (strain K105) were streaked or plated out on ISP medium 2, oatmeal agar (Hashimoto *et al.*, 1992), MS agar (mannitol plus soya flour) (Hobbs *et al.*, 1989), minimal medium (MM) agar containing 0.5% (w/v) mannitol as a carbon source (Hopwood *et al.*, 1985; Kieser *et al.*, 2000), R2 agar (Hopwood *et al.*, 1985; Kieser *et al.*, 2000), and modified SMMS agar as described Takano *et al.* (2000), and cultivated at 28^{0}C for 7 days for analyzing morphological difference.

4.2.7 Analysis of IM-2 production.

Spores of each strain (6.6 x 10^8 spores per 25 ml medium) were inoculated on ISP medium 2 agar plates and incubated at 28^{0}C for 19 h. Culture from four plates including agar were cut into small pieces and kept frozen at –80^{0}C for 1-h. Ethanol (60 ml) (adjusted to pH 2.0 with HCl) was added, and the supernatant was obtained by centrifugation, evaporated, and the residue extracted with ethyl acetate (20 ml). The ethyl acetate extract was evaporated and dissolved in methanol, clarified by passage through cotton, and the filtrates evaporated. The residue after evaporation was redissolved in methanol:H_2O (4:6) at a concentration of 200 mg per ml and purified in aliquots of 50 µl by HPLC (C_{18} reverse phase column; 10 mm x 250 mm; Cosmosil C_{18}) with methanol:H_2O (4:6) as a solvent and detection at 210nm. Fractions (19.8 ml-23.4 ml) corresponding to the elution position of authentic IM-2-C_4 were combined, evaporated, and dissolved in 3 ml of methanol for IM-2 bioassay. IM-2 activity in the sample was

assayed by measuring the IM-2 dependent production of BP (Yanagimoto and Enatsu, 1983). One unit of IM-2 activity is the minimum amount required for induction of BP production and corresponded to 0.6 ng (2.97 nM) of IM-2-C_5 per ml (Sato *et al.*, 1989). Authentic IM-2-C_4 was synthesized as described previously (Sato *et al.*, 1989).

4.3 Results and Discussion.

4.3.1 Insertional inactivation of *farA* in *S. lavendulae* FRI-5.

To assess the regulatory role(s) of FarA in secondary metabolism of *S. lavendulae* FRI-5, a 510 bp-*Bst*PI-*Pvu*II fragment internal to *farA* was replaced with a thiostrepton resistance gene (*tsr*), by which 170 amino acids corresponding to the 42nd to 211th amino acid of FarA (221 amino acids) were deleted. This deletion includes those constituting the second helix of helix-turn-helix DNA binding motif (32nd to 51th amino acid) in the N-terminus and the presumed autoregulator binding region in the C-terminal half (Sugiyama *et al.*, 1998), thus, the resulting truncated protein should be devoid of both the DNA binding and IM-2 binding activity.

The *farA* deletion allele together with each ca. 2.8 kb of 5'- and 3'-flanking region (Fig. 4.1) was cloned into pKC1132 (Fig. 4.2), a non-replicating plasmid in streptomycetes, to generate pSG104.

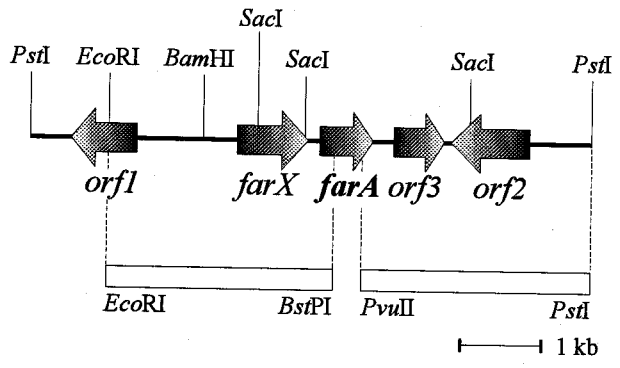

Fig. 4.1 Restriction endonuclease map of the *Pst*I-*Pst*I fragment (8.0 kb) around *farA* gene. This region contains four putative ORF other than *farA*. *farX* is a homologue of *afsA*, encoded with a putative enzyme for A-factor biosyntheis from *S. griseus*. *orf1*, *orf2* and *orf3* belong to SARP (*Streptomyces* antibiotics regulation protein) family, AraC family and is a acetyl transferase homologue, respectively.

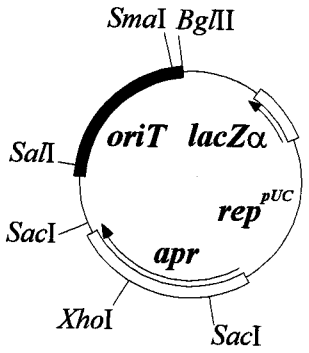

Fig. 4.2 Restriction endonuclease map of pKC1132. pKC1132 (3.5 kb) was derived from pOJ260,which is suicide vector for *Streptomyces oriT* RK2 for conjugation from *E. coli* to *Streptomyces,* and deleted with the *Kpn*I-*Spe*I fragment which contains the *Dra*I site from pOJ260.

Conjugal transfer from *E. coli* ET12567 (pUZ8002) harboring pSG104 to *S. lavendulae* FRI-5 gave apramycin-resistant exconjugants in which pSG104-integration by single crossover event was confirmed by Southern blot analysis (data not shown). After three rounds of sporulation cultivation of the pSG104-integrated strain in the presence of thiostrepton, thiostrepton-resistant and apramycin-sensitive colonies were obtained. Southern blot analysis of representative strains, such as strain K104, using PCR-amplified *farA* as a probe showed that a 2.1 kb-*Sac*I fragment in the wild type strain shifted to a 2.6 kb-band in strain K104 (Fig. 4.3), confirming that the K104 chromosome contained only the deleted *farA* gene by second crossover.

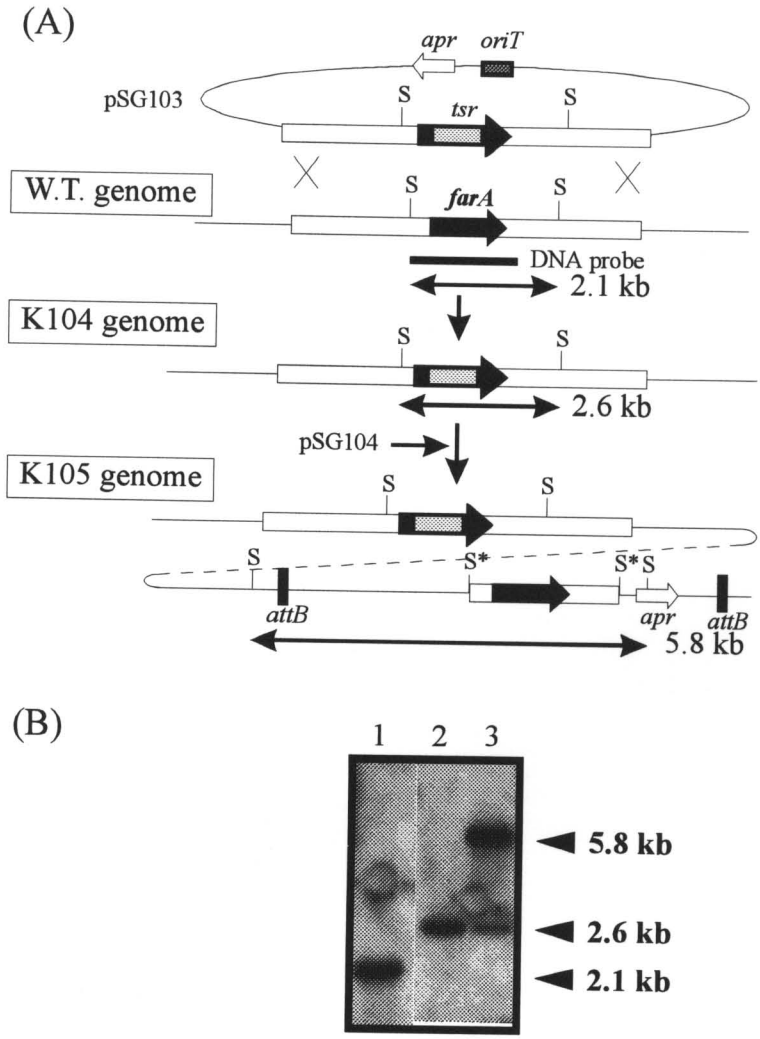

Fig. 4.3 Construction of a *farA* deletion mutant and its complemented strain. (A) Schematic representation of the strategy used for disruption of *farA* and its complementation. The solid arrow represents the *farA* gene, the gray arrow represents the thiostrepton resistance gene (*tsr*), the open arrow represents the apramycin resistance gene (*apr*), the crosshatched box represents the *oriT* sequence and the black box represents the ϕC31 attachment site (*attB* site). Abbreviations: S, *Sac*I; S*, *Sac*I site disrupted. (B) Southern hybridization analysis of chromosomal DNA from strain K101 (wild-type strain; lane 1), K104 (a *farA* deletion mutant; lane 2) and K105 (*farA* complemented strain derived from strain K104; lane 3) digested with *Sac*I. The probe used was the 0.9 kb PCR-amplified fragment containing the entire *farA* gene.

4.3.2 Phenotypic characterization of a *farA* deletion mutant (K104) in liquid culture.

To confirm the absence of a functional IM-2 receptor (FarA) in strain K104, IM-2 binding activity was measured with tritium-labeled IM-2-C_5 using crude extracts prepared from 8.5-h cells of strain K104 and the wild-type strain (Table 4.1).

Table 4.1 IM-2 binding activity of *S. lavendulae* FRI-5 wild-type strain(strain K101), a *farA* deletion mutant (strain K104), and a *farA* complemented strain (strain K105)

	IM-2 binding activity (pmol/mg-protein)	Percentage of control
Wild-type (K101)	1.55	100
farA deletion mutant (K104)	0.11	7.1
K104-complemented with intact *farA* (K105)	1.24	80

These values were highly reproducible.

The 8.5-h of cultivation was carefully selected, because the blue pigment production at 10.5-h in the wild-type strain indicates the presence at 8.5-h of endogenous IM-2 which usually precedes blue pigment production by about 2-h. The presence of endogenous IM-2 not only inhibits IM-2 binding assay by competing with labeled IM-2 but also enhances the amount of FarA in the wild-type strain by derepressing *farA* transcription (Waki *et al.*, 1997; Kitani *et al.*, 1999). The results of IM-2 binding assay demonstrated that strain K104 lost almost all IM-2 binding activity in comparison with a wild-type strain (strain K101).

To examine whether the *farA* mutation affects growth characteristics in liquid culture, growth of strains K101 and K104 were measured with or without exogenous addition of IM-2 at 5-h cultivation (Fig. 4.4.A and B). While the growth of the wild-type strain was repressed by the addition of synthetic IM-2-C_5 at a final concentration of 100 nM, strain K104 continued to grow irrespective of IM-2 addition, indicating that strain K104 became insensitive to the presence of IM-2.

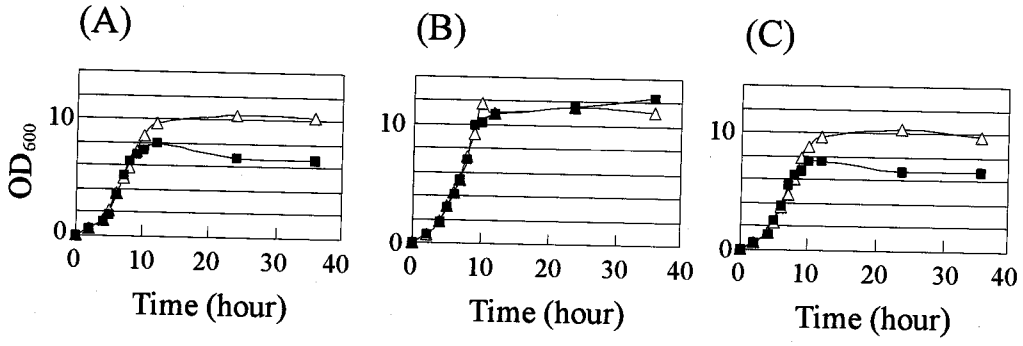

Fig. 4.4 Growth curves in liquid culture of (A) wild-type strain (strain K101), (B) a *farA* deletion mutant (strain K104) and (C) its *farA* complemented strain (strain K105). Each strain was grown in medium B at 28°C without IM-2 addition (open triangles) or with IM-2 addition (final 100 nM) at 5-h of cultivation (solid squares). Growth was monitored by measuring OD_{600}.

To evaluate how the *farA* deletion affects IM-2 signaling, the blue pigment (BP) production in strain K104 was initially monitored by measuring absorbance at 590 nm (Fig. 4.5.A).

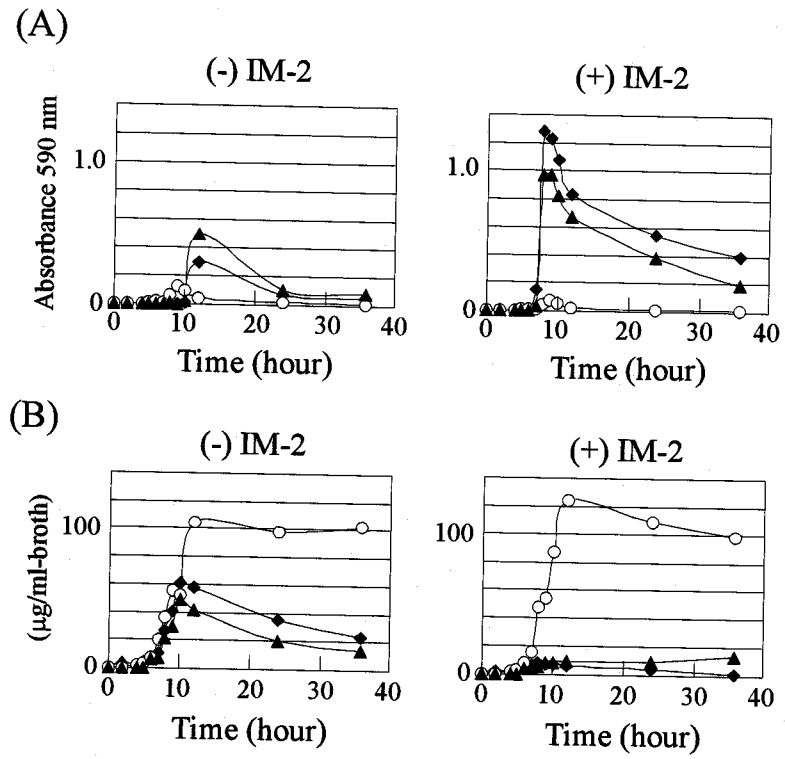

Fig. 4.5 Time courses of (A) blue pigment production and (B) D-cycloserine production in a *farA* deletion mutant and a wild-type strain. The amount of blue pigment and D-cycloserine at the indicated times were measured as described in Materials and Methods. Strain K101 (wild-type strain, solid lozenges, K104 (*farA* deletion mutant, open circles) and K105 (complemented strain, solid triangles) were grown at 28°C in medium B. (+) IM-2 above the figures indicates that exogenous IM-2 (final concentration of 100 nM) was added to the culture at 5-h of cultivation, and cultivation continued further. (-) IM-2 means no exogenous IM-2 addition.

In the wild-type strain (strain K101), without exogenous IM-2 addition, BP production was observed after 10.5-h cultivation, while addition of IM-2 at 5-h cultivation induced BP production from 7-h cultivation. In the *farA* mutant strain K104, however, regardless of IM-2 addition, BP production was observed from 8-h cultivation with much reduced levels of BP (about 4% to that of the wild-type strain with external IM-2). This phenomenon in strain K104 resembled that of the VB receptor-disruptants of *S. virginiae*, in which virginiamycin production began much earlier, if not constitutively, than did the parental strain but at an amount of only 10% of the parental strain (Nakano *et al.*, 1998; 2000).

Similar to BP production, production of nucleoside antibiotics [showdomycin (SHM) etc.] is induced by IM-2 in a wild-type strain (Hashimoto *et al.*, 1992). However, without the addition of IM-2, the production was small or negligible, yet external IM-2 clearly caused production (Hashimoto *et al.*, 1992), suggesting that the concentration of endogenously produced IM-2 (about 27 nM) was not high enough for the production and full activation of the biosynthesis of SHM, and its activation requires much higher concentrations of IM-2 generated by external IM-2 addition. In the *farA* deletion mutant strain K104, however, production of SHM was higher even without IM-2 addition than was that by the wild-type with the external IM-2 (Fig. 4.6), indicating that the *farA* deletion resulted in overproduction of nucleoside antibiotics. This phenomenon is similar to the case of an A-factor receptor (ArpA)-deficient mutant of *S. griseus*, in which a 10-fold final overproduction of streptomycin was observed with one-day earlier initiation of production than the wild-type strain (Miyake *et al.*, 1990), suggesting that *farA* is, as proposed for *arpA* in the streptomycin biosynthesis of *S. griseus*, the primary negative regulatory gene for the biosynthesis of nucleoside antibiotics in *S. lavendulae* FRI-5.

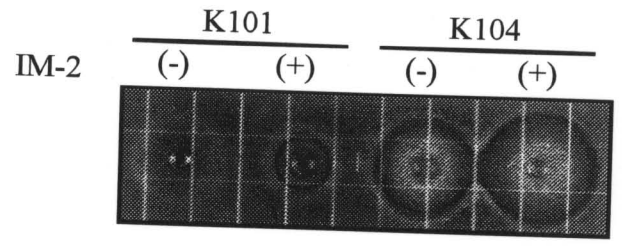

Fig. 4.6 Comparison of nucleoside antibiotics production between strain K101 and strain K104 with bioassay using *B. subtilis* PCI219. Supernatants from culture broth were applied onto active charcoal columns, absorbed compounds eluted with MeOH, concentrated, and used for bioassay. Each strain was cultivated for 31 h without (-) or with (+) IM-2 addition at 5-h of cultivation.

Although phenotypically positive effects on antibiotic production, such as those by IM-2 on nucleoside antibiotics and BP, are common among autoregulators, phenotypically negative effects on antibiotic production is unique among γ-butyrolactone autoregulators, and is seen only as the termination of D-cycloserine production by IM-2 (Hashimoto *et al.*, 1992). Because the repressor function seems common to all the autoregulator receptors (Kinoshita *et al.*, 1997; 1999; Onaka and Horinouchi, 1997; Kitani *et al.*, 1999), we hypothesized that FarA should repress a negative regulator of D-cycloserine production and expected that D-cycloserine production would be abolished in the *farA* mutant. However, strain K104 not only continued to produce DCS with pronounced increase of the production (Fig. 4.5.B), suggesting the presence of a completely different mode of regulation by FarA.

4.3.3 Phenotypic characterization of a *farA* deletion mutant on solid medium.

Recently, involvement of an A-factor receptor ArpA in the morphological control of *S. griseus* has been clearly demonstrated, in which ArpA indirectly represses transcription of *adsA* encoding extracytoplasmic function sigma factor necessary for morphological differentiation, via repressing *adpA* encoding an activator of the *adsA* (Yamazaki *et al.*, 2000). To clarify whether *farA* is involved in the morphological control of *S. lavendulae*,

2000). To clarify whether *farA* is involved in the morphological control of *S. lavendulae*, morphological characteristics of strains K101 and K104 were carefully compared on a range of different solid media. Because no differences in morphology or growth were detected between strains K101 and K104 (Fig. 4.7 and Fig. 4.8), the IM-2-FarA cascade was concluded to play no role in the morphological differentiation of *S. lavendulae* FRI-5.

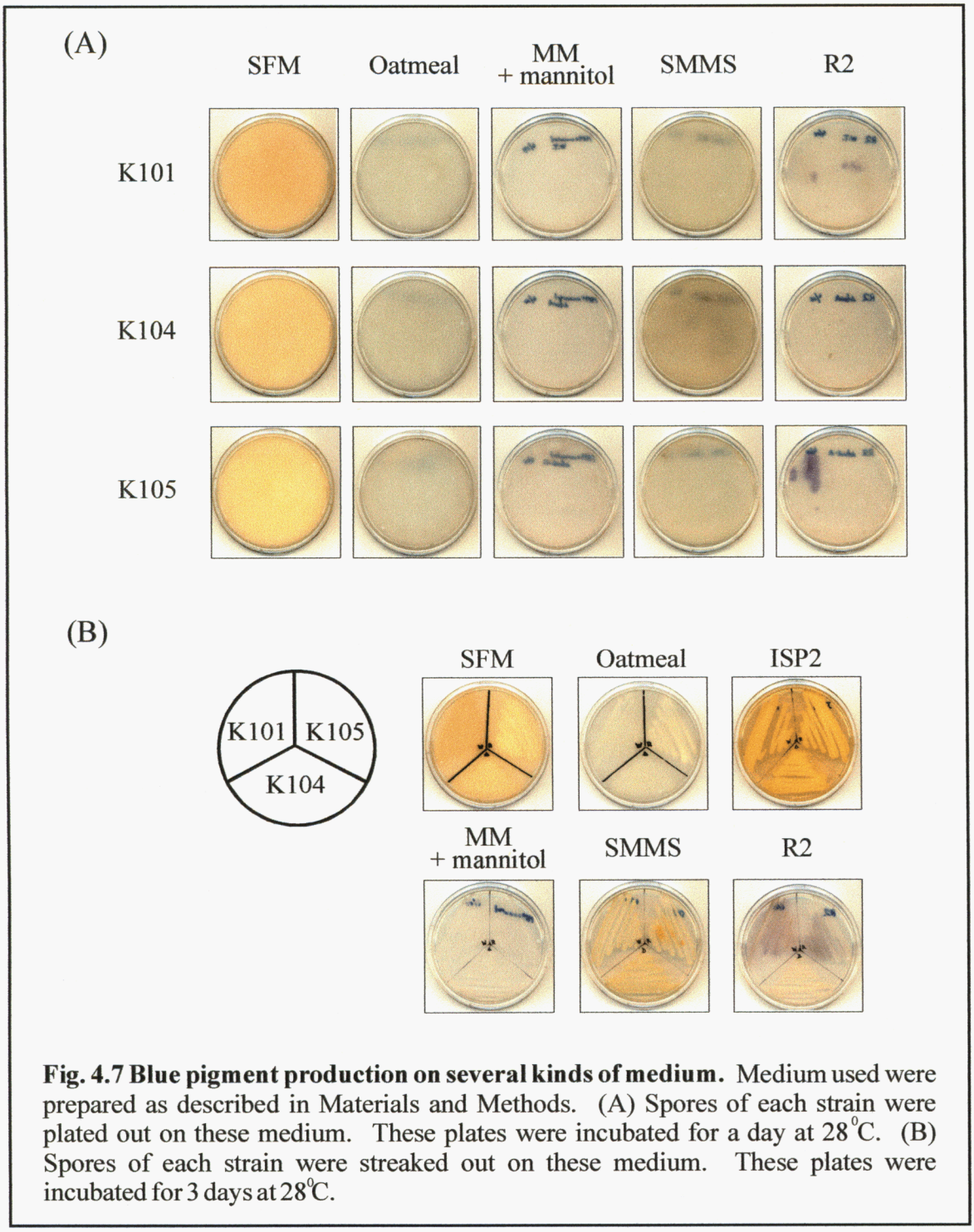

Fig. 4.7 Blue pigment production on several kinds of medium. Medium used were prepared as described in Materials and Methods. (A) Spores of each strain were plated out on these medium. These plates were incubated for a day at 28°C. (B) Spores of each strain were streaked out on these medium. These plates were incubated for 3 days at 28°C.

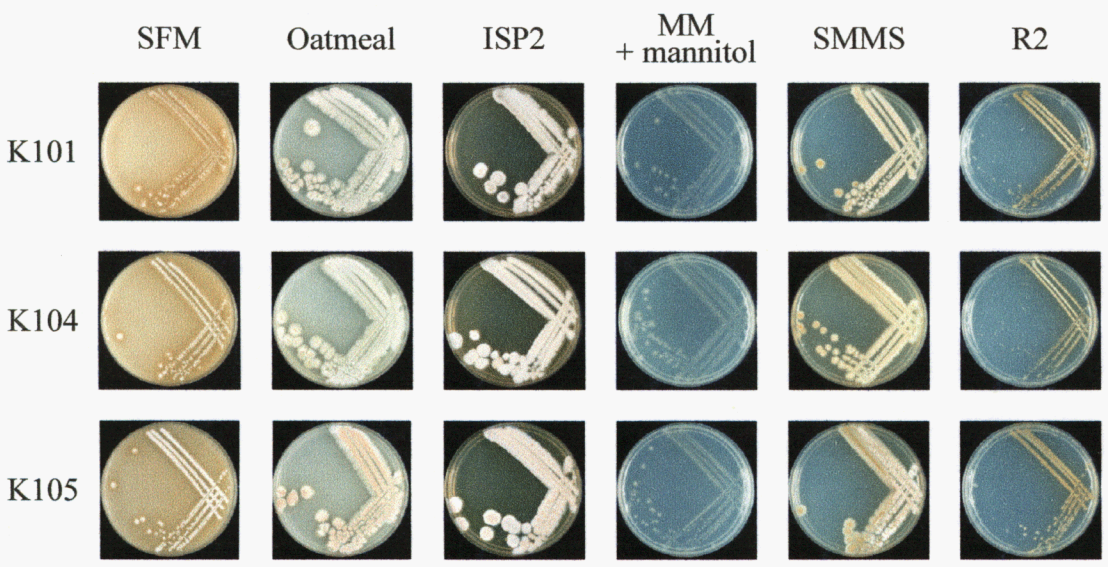

Fig. 4.8 Morpholgy on several kinds of medium. Medium used were prepared described in Materials and Methods. Spores of each strain were streaked out on these plates. These plates were incubated for 7 days at 28°C.

However, a wild-type strain with confluent lawn of growth produced a BP-like pigment on ISP medium 2 and R2 agar media, which was lacking or too small to be visible for strain K104 (Fig. 4.7 and Fig. 4.9), indicating that the phenomenon is FarA-dependent.

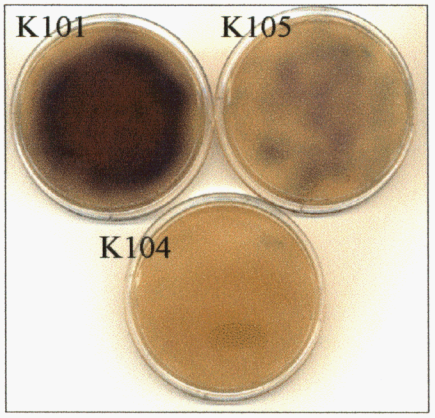

Fig. 4.9 Blue pigment production by strains K101, K104 and K105 on ISP medium 2. Spores of each strain were plated out on ISP medium 2, and plates were incubated for 19-h at 28°C.

Because this phenomenon can be considered to be IM-2-triggered onset of secondary metabolism on solid media, we assessed the concentration of IM-2 in the solid media

(Table 4.2).

Table 4.2 IM-2 production of *S. lavendulae* FRI-5 wild-type strain (strain K101), a *farA* deletion mutant (strain K104).and a *farA* complemented strain (strain K105)

	IM-2 produced by surface-grown culture (nM)	Percentage of control
Wild-type (K101)	6.48	100
farA deletion mutant (K104)	0.71	11
K104-complemented with intact *farA* (K105)	7.13	110

These values were highly reproducible.

As expected, wild-type strain (K101) was found to produce 6.48 nM of IM-2 which was 2.2-fold higher than the minimum effective concentration of IM-2 (2.97 nM). Surprisingly, strain K104 produced only 11% of IM-2 to that of wild-type strain, indicating that FarA should be necessary for IM-2 biosynthesis. Because similar phenomenon that intact autoregulator receptor is required for the production of corresponding autoregulator has been also observed in the BarA null mutant (Nakano *et al.*, 2000) of *S. virginiae* and the SCB1 receptor (ScbR) mutant of *S. coelicolor* A3(2) (Takano and Bibb, personal communication), autoregulator receptor may generally be involved in the regulation of autoregulator biosynthesis.

4.3.4 *cis* complementation of a *farA* deletion mutant.

To confirm that phenotypic differences observed between the wild-type strain and the *farA* mutant (strain K104) were due to the lack of functional FarA protein, an intact *farA* gene was transconjugated and integrated into an *attB* site of strain K104 via pSG105, a derivative of a *Streptomyces* integration plasmid pSET152 containing ϕC31 *attP* site and

int. The integration of the intact *farA* in apramycin-resistant exconjugants was confirmed by Southern hybridization (Fig. 4.3), and designated as strain K105. All the characteristics of strain K105 [the IM-2 binding activity (Table 4.1), growth in the presence of IM-2 (Fig. 4.4.C), BP production (Fig. 4.5.A), D-cycloserine production (Fig. 4.5.B), SHM production (data not shown), and IM-2 production (Table 4.2)] were restored to the wild-type phenotypes. Furthermore, pigment production on solid media (ISP medium 2 and R2 agar) was also restored in strain K105 (Fig. 4.7 and Fig. 4.8), suggesting that it is a FarA-dependent pigment production on solid media, similar to the FarA-dependent BP production in liquid media. These lines of evidence clearly demonstrated that the phenotypic changes in strain K104 were due solely to the loss-of-function of *farA*.

4.4 Summary.

I constructed a *farA* deletion mutant by means of homologous recombination to clarify the in vivo function of an IM-2 specific receptor (FarA) in the IM-2-triggered signaling cascade in *S. lavendulae* FRI-5. On several solid media, no significant difference in morphology was observed between the wild-type strain and the *farA* deletion mutant (strain K104), which demonstrated that the IM-2-FarA system does not participate in morphological control of *S. lavendulae* FRI-5. In liquid media, the *farA* mutant overproduced nucleoside antibiotics and produced the blue pigment earlier than did that by the wild-type strain, establishing that FarA protein primarily acts as a negative regulator on the biosynthesis of these compounds in the absence of IM-2. However, contrary to the IM-2-dependent suppression of D-cycloserine production in the wild-type strain, overproduction of D-cycloserine was observed in the *farA* mutant, suggesting that the FarA-IM-2 complex, not the unbound FarA, should be essential in the negative control of the D-cycloserine biosynthesis.

Chapter 5

General conclusions

Members of the filamentous, Gram-positive bacterial genus *Streptomyces* are versatile producers of many secondary metabolites, including over two-thirds of all antibiotics used in human medicine and in agriculture (Alderson *et al.*, 1993; Berdy *et al.*, 1995). In recent years, there have been rapid advances in our understanding of the mechanisms that regulate antibiotic production and/or morphological differentiation in streptomycetes. Among the factors known to affect these characteristics, γ-butyrolactone autoregulators have been shown in several streptomycetes to serve as extracellular signaling molecules determining the onset of the two noteworthy characteristics. These autoregulators have a common 2,3-disubsituted γ-butyrolactone skeleton, and 10 compounds have been identified to date.

IM-2, one of the autoregulators, is produced by *Streptomyces lavendulae* FRI-5 and has the unique function of being both a positive and a negative mediator with respect to the production of antibiotic nucleoside antibiotics, blue pigment, and D-cycloserine during the stationary phase. Since other autoregulators have only positive function in relation to antibiotic production or morphological differentiation, elucidation of this switching mechanism is very important for acquiring knowledge on more general but more complicated autoregulator-dependent pathways in the *Streptomyces* species. As a first clue in clarifying the regulation mechanisms, the in vitro and in vivo analysis of autoregulator receptors is most important. A recent study on *S. griseus* came close to establishing a model for the A-factor regulatory cascade leading to the onset of streptomycin production and morphological differentiation, and this is likely to represent the simplest model (Ohnishi *et al.*, 1999; Yamazaki *et al.*, 2000). However, in *S.*

lavedulae FRI-5, nothing is known except that an IM-2 specific receptor (FarA) protein is present in this strain.

In this thesis project, I analyzed the DNA-binding activity of FarA in vitro, determined the binding sequence, and established the method of introducing DNA in *S. lavendulae* FRI-5. Furthermore, I clarified the in vivo function of FarA by constructing and analyzing a *farA* disruption mutant, and based on the above, have proposed a model for the IM-2-mediated signaling cascade in the secondary metabolism of *S. lavendulae* FRI-5. The results are briefly described as follows:

As described in chapter 2, as a first step in clarifying the FarA-dependent regulation mechanism, the DNA-binding activity of FarA was verified by in vitro techniques. Gel-shift assays demonstrated that FarA binds to the *farA* upstream region and that this binding is abolished in the presence of IM-2. The FarA binding sequence was localized by DNase I footprinting to a 28 bp sequence located approximately 70 bp upstream of the *farA* translational start site. High-resolution S1 nuclease mapping of *farA* transcripts revealed a putative transcription start site located at an A residue positioned 64 bp upstream from the *farA* translation start codon and 4 bp downstream from an *Escherichia coli* σ^{70}-like -10 recognition region. The FarA binding sequence (FARE, FarA-responsive element) overlaps this -10 region and contains the *farA* transcription initiation site, suggesting that FarA acts as a repressor that, in the absence of IM-2, represses transcription of *farA*.

As described in chapter 3, since the development of a means to introduce DNA into the organism is very important to acquiring further knowledge regarding the in vivo function of FarA, I have established a reliable system for the intergeneric transfer of DNA from *E. coli* ET12567 (pUZ8002) to *S. lavendulae* FRI-5, and have determined the optimal conditions using pSET152 on two kinds of media (MS agar or ISP2 containing 10

mM $MgCl_2$). These results suggest that the frequencies of conjugation in this strain depend on the kinds of media and the length of DNA to be introduced. Germination of recipient spores prior to mixing with the *E. coli* donor did not increase the frequency of conjugation. I also confirmed that chromosomal integration occurs at a unique ϕC31 *attB* site and does not influence morphological differentiation or IM-2-dependent signal transduction. Importantly, this procedure will allow targeted gene disruption and mutant complementation in *S. lavendulae* FRI-5, enabling us to investigate the complex regulatory cascade of IM-2-dependent regulation on secondary metabolism in this strain.

As described in chapter 4, to clarify the in vivo function of FarA protein, I constructed a *farA* deletion mutant (strain K104) by homologous recombination with the established conjugation method described in chapter 3. On several solid media, no significant difference in morphology was observed between the wild-type strain (K101) and the *farA* mutant, demonstrating that the IM-2-FarA system does not participate in morphological control in *S. lavendulae* FRI-5. In liquid media, the *farA* mutant overproduced nucleoside antibiotics and produced the blue pigment earlier than that by the wild-type strain, establishing that the FarA protein primarily acts as a negative regulator on the biosynthesis of these compounds in the absence of IM-2. However, contrary to the IM-2-dependent suppression of D-cycloserine production in the wild-type strain, overproduction of D-cycloserine was observed in the *farA* mutant, suggesting that the FarA-IM-2 complex, not the unbound FarA, is essential to the negative control of D-cycloserine biosynthesis.

I propose the following model for the IM-2-mediated signaling cascade in the secondary metabolism of *S. lavendulae* FRI-5 (Fig. 5.1).

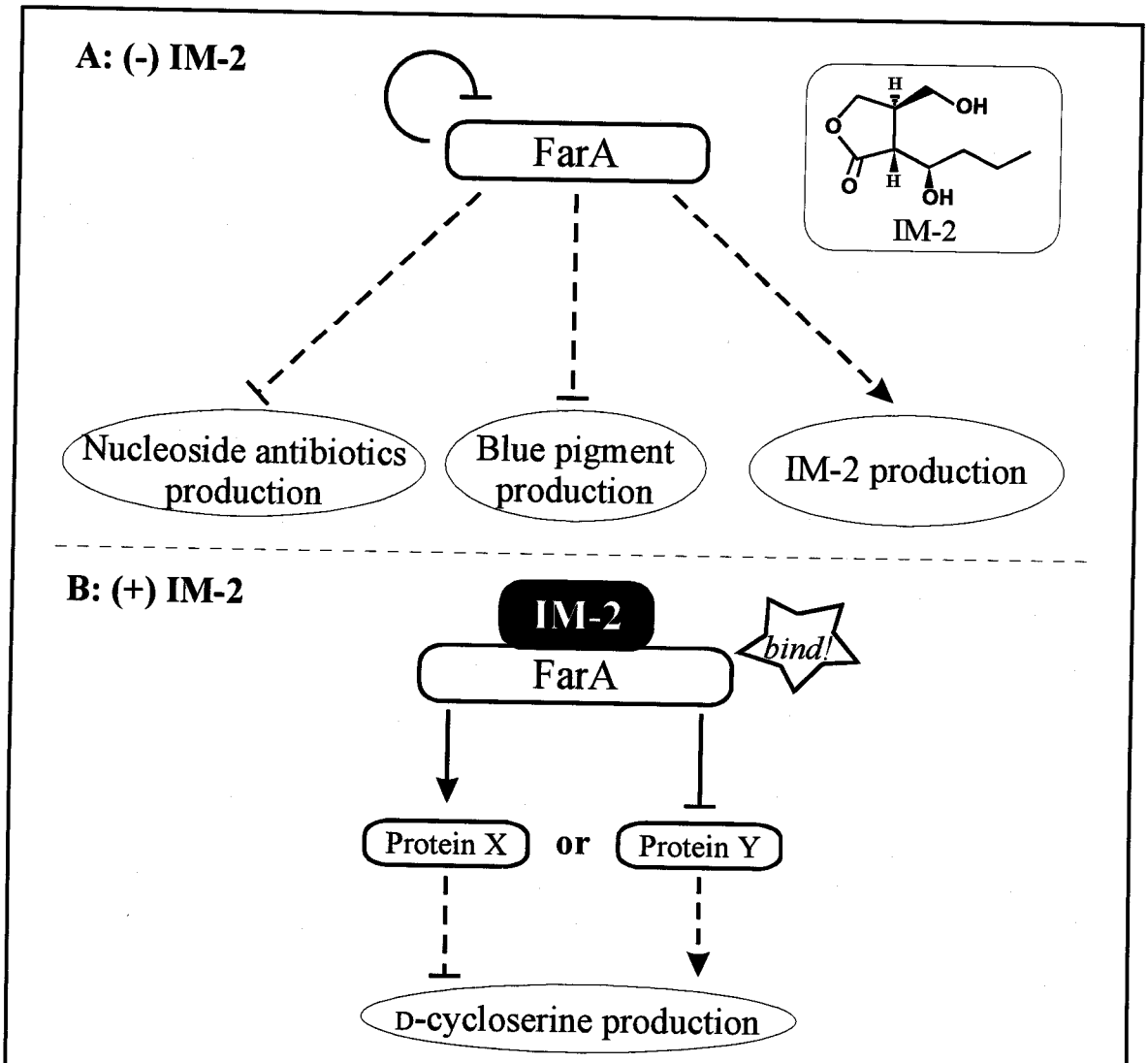

Fig. 5.1 The IM-2 regulatory cascade leading to production of secondary metabolism in the absence (A) or presence (B) of IM-2. FarA should act as a repressor on the biosyntheis of nucleoside antibiotics and blue pigment. On the other hand, FarA-IM-2 complex should act as an regulator on triggering termination cascade of D-cycloserine biosynthesis.

At first, FarA acts as a transcriptional repressor on *farA* itself, forming an autoregulatory circuit that should serve to sense and maintain intracellular free IM-2 concentrations under some threshold level in the cells. This autoregulatory circuit has also been observed in the VB-BarA system of *S. virginiae* (Kinoshita *et al.*, 1997; 1999) and in the recently found SCB1-ScbR system of *S. coelicolor* A3(2) (Takano and Bibb, personal communication); this circuit thus seems to be common to γ-butyrolactone autoregulator-

receptor systems of streptomycetes. Next, FarA should be considered to regulate biosynthesis of IM-2 itself from the phenomenon that IM-2 production was dramatically decreased in the *farA* disruptant. Thirdly, the biosyntheses of both blue pigment and nucleoside antibiotics appear to be negatively controlled by FarA. However, there exists a difference in their degrees of regulation. FarA seems to be a primary/dominant negative regulator in the biosynthesis of nucleoside antibiotics, with its disruption resulting in an overproduction of nucleoside antibiotics, a phenomenon identical to that in ArpA-streptomycin production in *S. griseus* (Miyake *et al.*, 1990). In contrast, FarA is not the dominant regulator in the biosynthesis of blue pigment, so that its disruption resulted in earlier initiation but with the amount of blue pigment being much reduced compared to that with the wild-type strain. *S. lavendulae* should have an additional regulatory mechanism that terminates premature initiation of blue pigment, a phenomenon similar to that in BarA-virginiamycin production in *S. virginiae* (Nakano *et al.*, 1998; 2000). Finally, and most striking feature of the *farA* mutant was found to be the overproduction of D-cycloserine, which was completely opposited to the expected none-production of D-cycloserine. Supposing that IM-2-unbound FarA is either repressing a repressor gene or activating an activator gene of D-cycloserine biosynthesis, the loss-of-function mutation of *farA* should result in a loss of DCS production. Therefore, this overproduction phenomenon is unexplainable if unbound FarA is the only functional form. Instead, the IM-2-FarA complex should be considered to act as a regulator that triggers the termination cascade of DCS biosynthesis (Fig. 5.B). Similar data suggesting the involvement of the receptor-autoregulator complex in the regulation of antibiotic production have recently been obtained from the analysis of *S. coelicolor* A3(2) M752, a disruptant of the SCB1 receptor (ScbR), in which SCB1-induced undecylprodigiosin production is noticeably delayed compared to the parental strain, M145 (Takano and Bibb,

personal communication). It should be clarified in the future whether IM-2-FarA complex is activating a repressor gene or repressing an activator gene of DCS biosynthesis. Because genes under the control of the IM-2-FarA complex are expected to be bound by the IM-2-FarA complex, I am currently searching for DNA fragments bound by FarA in the presence of IM-2, a process that should provide clues toward further clarifying the signaling mechanism in *S. lavendulae* in particular and to understand, in general, the γ-butyrolactone-dependent regulatory cascade in streptomycetes.

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Related Publications

1) In vitro analysis of the butyrolactone autoregulator receptor protein (FarA) of *Streptomyces lavendulae* FRI-5 reveals that FarA acts as a DNA-binding transcriptional regulator that controls its own synthesis.

Shigeru Kitani, Hiroshi Kinoshita, Takuya Nihira, and Yasuhiro Yamada

Journal of Bacteriology (1999) **181:** 5081-5084

2) Conjugal transfer of plasmid DNA from *Escherichia coli* to *Streptomyces lavendulae* FRI-5.

Shigeru Kitani, Mervyn J. Bibb, Takuya Nihira, and Yasuhiro Yamada

Journal of Microbiology and Biotechnology (2000) **10:** 535-538

3) Gene replacement analysis of the butyrolactone autoregulator receptor (FarA) reveals that FarA acts as a dual-function regulator in secondary metabolism of *Streptomyces lavendulae* FRI-5.

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