Pleiotropic control of secondary metabolism and morphological development by KsbC, a butyrolactone-autoregulator receptor homologue in *Kitasatospora setae*

（放線菌 *Kitasatospora setae* におけるオートレギュレーターレセプター型制御因子 KsbC の二次代謝及び形態分化への関与）

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September 2012

Division of Advanced Science and Biotechnology,

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Chapter 1

General Introduction

1.1 The genera of actinomycetes, the fascinating microorganisms producing useful secondary metabolites

Actinomycetes are a large group of phylogenetically related, filamentous and aerobic gram-positive bacteria, showing high GC content in their genetic materials. The actinomycetes typically have two features. The more striking of these two characteristics is a complex cycle of morphological differentiation, including germination of spores, elongation and branching of substrate mycelia, formation of aerial mycelia, and septation of hyphae and spore maturation (Fig. 1.1) (Chater and Losick, 1997). Surface-grown *Streptomyces* colonies may be considered multicellular organisms with several distinct cell types. The spores exist in a semi-dormant stage in the life cycle and can survive in this stage in soil for long periods (Mayfield et al., 1972; Ensign, 1978). The spores confer resistance to low nutrient and water availability, whereas the mycelial stage is sensitive to drought (Karagouni et al., 1993). Spore germination requires exogenous nutrients, water and Ca^{2+} (Ensign, 1978), and the nutrient status of the germination site limits the extent of hyphal growth and the time to differentiation into aerial hyphae and eventually spores (Wellington et al., 1990).

The other characteristic is that members of actinomycetes are resourceful in their production of bioactive compounds as secondary metabolites. Among the antibiotics derived from microbial sources, actinomycetes provide as much as 70% of the antibiotics used in human therapy, with the other 30% being produced from other bacteria and fungi (Bérdy, 2005). The majority of the actinomycetes belong to the genera *Streptomyces*, which are well-

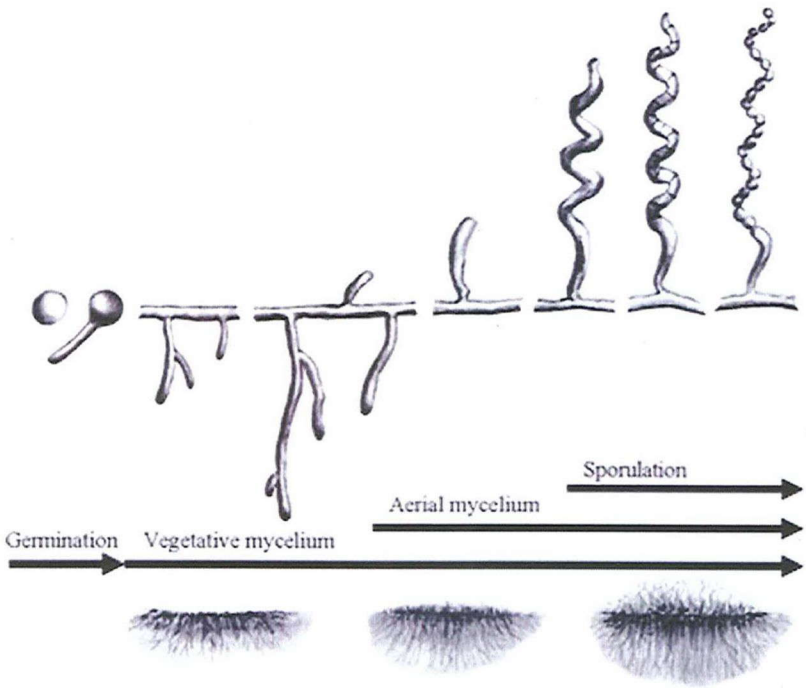

Fig. 1.1 The development life cycle of *Streptomyces coelicolor*. Schematic drawing by Markus Hempel, adapted from originals by K. F. Chater.

known for their abilities to produce diverse and enormous bioactive metabolites. In particular, in the "golden era" of antibiotic discoveries in the forties and early fifties, the newly discovered antibiotics were mainly isolated from *Streptomyces* species, which represented some 70% to 80% of the all isolated compounds. However, in the next two decades the significance of the non-*Streptomyces* actinomycetes species (rare actinomycetes) increased, with these species making up 25-30% of all antibiotics (Fig. 1.2) (Bérdy, 2005). In Table 1 the numbers of *Actinomycetales* species known to produce bioactive metabolites, including the rare actinomycetes, are summarized. These fastidious organisms, the rare actinomycetes, produce a group of unique, highly diverse and sometimes very complicated compounds that exhibit excellent antibacterial potency and usually low toxicity. Within this group of metabolites there are numerous practical and very important compounds, such as gentamycins, erythromycins, vancomycin, and rifamycin. Numerous recently introduced

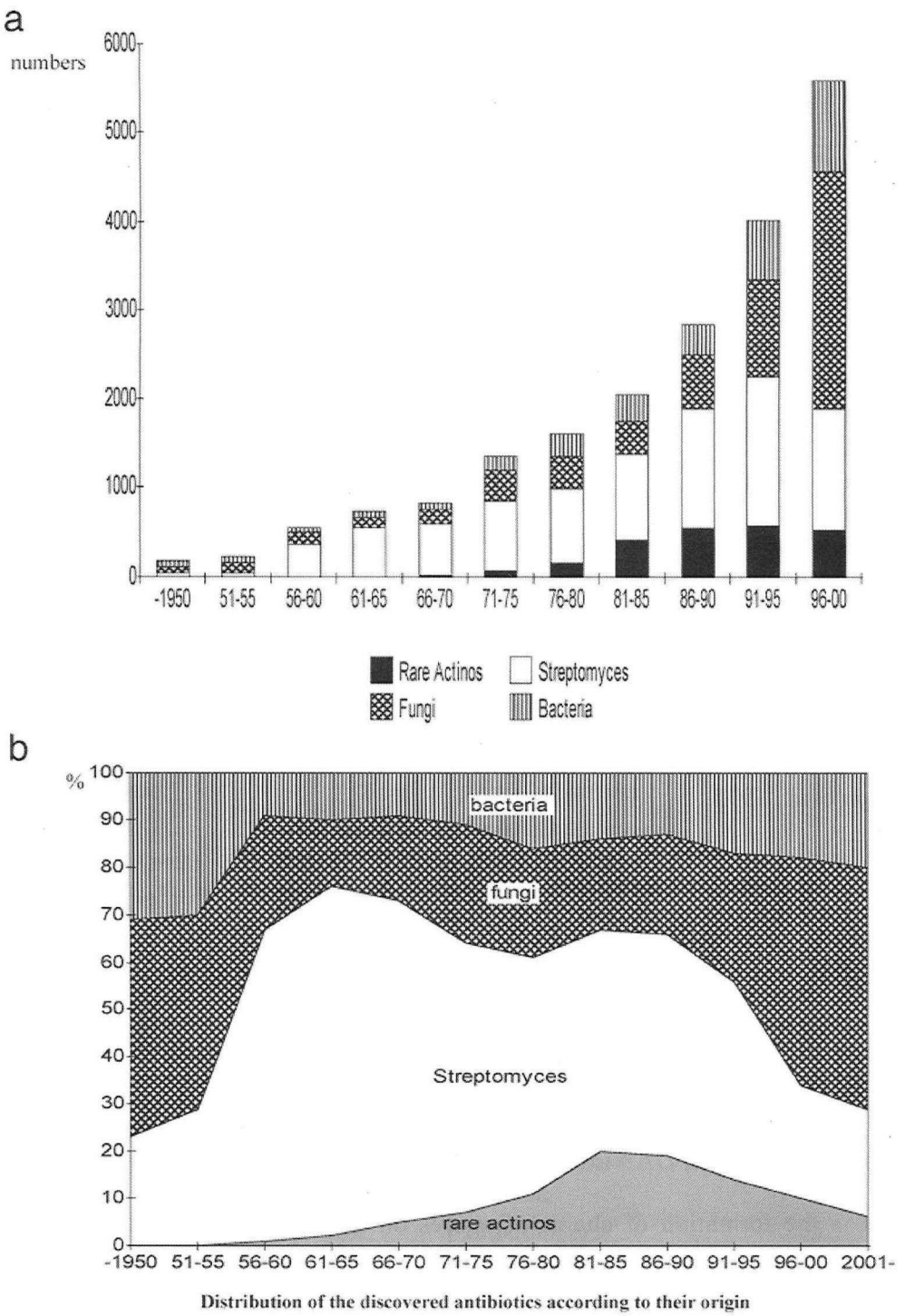

Fig. 1.2 Distribution of the discovered antibiotics according to their origin (Bérdy, 2005).

chemotherapeutic and agricultural agents (ziracin, dalbavacin, spynosin) are also rare actinomycete products. It is noteworthy that the vancomycin-ristocetim-type complicated

glycopeptides are produced almost exclusively by various rare actinomycete species (Bérdy, 2005).

A whole array of taxonomic tools has been used to define genera and suprageneric groups of actinomycetes (Goodfellow, 1989). However, the major emphasis is now on 16S rDNA homologies, in addition to cell wall analysis and fatty acid and lipid patterns (Williams et al., 1989; Wellington et al., 1992). One of the quickest methods for preliminary identification at the genus level has been the presence of the LL and/or the meso isomer of diaminopimelic acid (DAP) as the diamino acid in the peptidoglycan. This feature, when combined with the characteristic substrate and aerial mycelium, is simply diagnostic for the classification of *Streptomyces* and non-*Streptomyces* (Goodfellow and Pirouz, 1982). However, it was later shown that some strains that were previously classified as *Kitasatosporia* may contain major amounts of LL-DAP in the vegetative mycelium only (Wellington et al., 1992).

1.1.1 The genus *Streptomyces*

The order *Actinomycetales* is divided into groups according to the composition of the cell wall. Within each group, the morphology of the aerial mycelium, the spore arrangement, and the chemical characteristics are used to define the genus. *Streptomyces* belongs to the group containing only LL-DAP in the cell wall. Sometimes, the distinctive characteristic of the genus is the formation of chains of spores on the aerial mycelium. The spores are maintained in chains by a sheath that confers to them various types of surfaces: smooth, spiny, hairy, etc. The chains may be straight or flexuous, or may form open or closed spirals. The shape of the chains and the appearance of the spores are elements used for the species determination. Other characteristics that are considered include the color of the surface and reverse of colonies, growth above or below 45°C, utilization of carbon compounds, and

Table 1 Number of *Actinomycetales* species producing bioactive microbial metabolites (Bérdy, 2005)

Streptomycetaceae:		*Thermomonosporaceae*:	
Streptomyces	~8,000	*Actinomadura*	345
Streptoverticillium	258	*Saccharothrix*	68
Kitasatosporia	37	*Microbispora*	54
Chainia	30	*Actinosynnema*	51
Microellobosporia	11	*Nocardiopsis*	41
Nocardioides	9	*Microtetraspora/Nonomuria*	26/21
		Thermomonospora	19
Micromonosporaceae:		*Micropolyspora/Faenia*	13/3
Micromonospora	740	*Thermoactinomyces*	14
Actinoplanes	248	*Thermopolyspora*	1
Dactylosporangium	58	*Thermoactinopolyspora*	1
Ampullariella	9		
Glycomyces	2	*Mycobacteriaceae*:	
Catenuloplanes	3	*Nocardia*	(357)
Catellatospora	1	*Mycobacterium*	57
		Arthrobacter	25
Pseudonocardiaceae:		*Brevibacterium*	17
Saccharopolyspora	131	*Proactinomyces*	14
Amycolatopsis/Nocardia	120/357	*Rhodococcus*	13
Kibdellosporangium	34		
Pseudonocardia	37	Other (unclassified) species:	
Amycolata	12	*Actinosporangium*	30
Saccharomonospora	2	*Microellonosporia*	11
Actinopolyspora	1	*Frankia*	7
		Westerdykella	6
Streptosporangineae:		*Kitasatoa*	5
Streptosporangium	79	*Synnenomyces*	4
Streptoalloteichus	48	*Sebekia*	3
Spirillospora	11	*Elaktomyces*	3
Planobispora	10	*Excelsospora*	3
Kutzneria	4	*Waksmania*	3
Planomonospora	2	*Alkalomyces*	1
		Catellatospora	1
		Erythrosporangium	1
		Streptoplanospora	1
		Microechinospora	1
		Salinospora	1

solubilization of specific substrates (Kieser et al., 2000).

Among the actinomycetes the genus *Streptomyces* is most abundant (above 95% of the whole actinomycetes) (Lechervalier and Lechervalier, 1967) and well-studied. *Streptomyces* hyphae are 0.5 to 2 μm thick. Nucleoids are distributed along the entire length of the hyphae, and a very high G+C content of 69-78% is characteristic of the genus. The overall cell wall composition is similar to that of other gram-positive bacteria; i.e., the peptidoglycan chains are joined by glycine bridges. Teichoic acids are also present but mycolic acids are absent. Menaquinones have chains of nine isoprene units, with three or four of the double bonds saturated. Linear saturated and branched fatty acids are present in the membrane lipids, with the iso-16 and anteiso-15 and -17 usually predominating.

The complex life cycle of *Streptomyces* species is one of the major reasons for studying their genetics (Fig. 1.1). During the growth of a colony from a spore, colonial complexity increases (Hodgson, 1992; Chater, 1993; Champness and Chater, 1994; Chater and Losick, 1997). After a suitable germination trigger, the germ tube emerges from the unigenomic spore and its cell wall grows mainly at the tip, at a rate that doubles with each replication of the chromosomes in the initially aseptate germ tube (Chater and Losick, 1997). The potentially catastrophic acceleration rate of tip extension becomes stabilized after a few DNA replication cycles; this is because new tips arise through lateral branching as further doublings in chromosome number take place. Many branches are close to septa. Both branches and septa are most frequent in older parts of the mycelium, and are not found very close to tips. The formation of septa at some distance behind tips produces an apical and a sub-apical compartment. The sub-apical compartments differ from the apical compartments in various respects: they lack new cell wall synthesis, but DNA synthesis nevertheless takes place. If a branch forms in a sub-apical cell, it regains some apical properties.

It is well known that members of the genus *Streptomyces* produce a great many antibiotics and other classes of biologically active secondary metabolites. Indeed, as shown in Fig. 1.2, actinomycetes make up some two-thirds of the known antibiotics that are produced by microorganisms, and amongst them about 74% are made by members of the genus *Streptomyces*.

1.1.2 The genera of non-*Streptomyces* actinomycetes

More than 95% of all actinomycetes isolated from nature are of the genus *Streptomyces* (Lechervalier and Lechervalier, 1967); the other 5% or less are made up of non-*Streptomyces* actinomycetes, sometimes called "rare" actinomycetes. Recently, non-*Streptomyces* actinomycetes have received attention as very rich sources of medically important secondary metabolites and new antibiotics. Since the discovery of streptomycin, a large number of antibiotics, including major therapeutic agents such as amino glycosides, chloramphenicol, tetracyclins, macrolides and more recently the β-lactam cephamycin group, have been isolated from cultures of *Streptomyces* and used in human medicines. The increases in infections caused by antibiotic-resistant bacteria and new human diseases are currently creating new challenges in the field. In order to solve these problems, the focus of industrial screening and study for discovering new and excellent bioactive microbial products including antibiotics has therefore moved to less exploited non-*Streptomyces* actinomycetes strains, such as *Actinomadura*, *Micromonospora*, *Actinoplanes*, *Nocardia*, *Saccharopolyspora*, and *Amycolatopsis*. These species have statistically greater potential as producers of bioactive products (over 26% of all bioactive microbial metabolites produced by actinomycetes) than *Streptomyces* species, despite their uneven distribution ratio (Lazzarini et al., 2000). In addition, the genera of non-*Streptomyces* actinomycetes have more diverse chemotaxonomic and morphologic characteristics than the genus *Streptomyces*. In this section, the

characteristics of non-*Streptomyces* actinomycetes belonging to the families *Micromonosporaceae*, *Nocardiaceae*, *Pseudonocardiaceae*, *Streptosporangiaceae* and *Thermomonosporaceae* are summarized. For the details of *Kitasatospora setae*, a model strain in this study, which is one of the non-*Streptomyces* actinomycetes, however, they are described in section 1.2 together with the genome information of the strain.

1.1.2.1 The family *Micromonosporaceae*

The group of *Micromonosporaceae* comprises actinomycetes producing motile spores enclosed in vesicles called sporangia. However, the genus *Micromonospora*, which bears nonmotile single spores, is also included in this group, because of similar chemical traits and the homology of nucleic acids. *Micromonosporaceae* are gram-positive organisms, growing with branched septated hyphae; the aerial mycelium is rarely developed and never abundant. The peptidoglycan of the cell wall contains meso-DAP or 3-hydroxydiaminopimelic acid and glycine rather than L-alanine. In the cell membrane, iso- and anteiso-fatty acids predominate. Although the production of motile spores suggests an adaptation to aquatic habitats, *Micromonosporaceae* are found in any type of soil. They are aerobic and mesophilic, growing well between 20°C and 30°C and at a pH around 7.

Acinoplanes. Colonies of *Acinoplanes* are small, reflecting the slow growth of these organisms. The vast majority have a characteristic orange color, although brown, red, and blue strains have been described. The color is the result of carotenoid pigments. The sporangia are subspherical, often borne on stalks called sporangiophores. Spore flagella are polar. *Actinoplanes* strains can grow on minimal media, in which sporulation is favored, but they are usually cultured in the rich media generally recommended for actinomycetes. Typically, they utilize xylose or chitin as carbon sources; the enzyme xylose isomerase from *A. missourinensis* is used industrially for the conversion of glucose to fructose (Vobis, 1992).

More than 240 antibiotics have been isolated from *Actinoplanes* strains (Table 1). Amino acid derivatives, such as peptides and depsipeptides, are prevalent, but polyene, aromatic, nucleoside, and chloro-heterocyclic compounds are also produced. Of clinical relevance is the lipoglycopeptide teicoplanin, used in the treatment of gram-positive infection and isolated as a complex of five components from *A. teichomyceticus* cultures. Of potential therapeutic interest is ramoplanin, a macrocyclic peptide with sugar and fatty acid substituents, isolated from *Actinoplanes* sp. ATCC33076. Other products of interest are the pleuracins, mixtures of macrocyclic lactones and depsipeptides that are closely related to the streptogramins group and are isolated from *A. auranticolor* and *A. azureus*; lipiarmycin, an unusual macrolide inhibitor of RNA polymerase produced by *A. deccanenensis*; and purpuromycin, a polyketide with a broad spectrum of antibacterial and antifungal activity produced by *A. ianthinogenes* (Parenti and Coronelli, 1979). Acarbose, a tetra-pseudosaccharide composed of three sugars and an aminocyclitol produced by *Actinoplanes* sp., is a selective inhibitor of the gastric α-amylase and sucrase. It has been proposed for use in the treatment of metabolic disorders as an antihyperglycemic drug, since it delays or hinders the intestinal absorption of carbohydrates (Brunkhorst et al., 1999).

Micromonospora. Colonies of *Micromonospora* strains are macroscopically similar to those of *Actinoplanes*, showing the same orange color, but can be easily distinguished microscopically by the absence of sporangia. Aerial mycelia are absent; spores are borne on sporophores on the vegetative mycelium and are often also observed in liquid cultures. Chemical characteristics are similar to those of *Acinoplanes*. Cultures require neutral pH; common sugars and starch are suitable sources of carbon, with ammonia or amino acids serving as nitrogen sources. The preferred habitats are lake mud and river sediments, where they are sometimes the only actinomycetes present (Luedemann, 1969; Suarez and Hardisson, 1985).

Over 700 antibiotics have been described from *Micromonospora* strains (Table 1). The most important ones are as follows: the aminocyclitols, such as the gentamicins, produced by *M. purpurea* and *M. echinospora*; sisomicin from *M. inyonensis*; and fortimicin from *M. olivoasterospora*. Of practical interest are the macrolides rosamicin, from *M. rosaria*, and the mycinamicins, isolated from *M. griseorubida*. A family of macrolides closely related to lipiarmycin, the clostomicins, is produced by *M. echinospora*. Ansamycins of the rifamycin family have been isolated from *M. halophytica*. The polysaccharide everninomycin is a typical product of *M. carbonacea* (Wagman and Weinstein, 1980).

1.1.2.2 The families *Nocardiaceae* and *Pseudonocardiaceae*

These families make up a very heterogeneous group that includes actinomycetes that form a mycelium breaking up into coccoid or rod-shaped fragments. All of the organisms are gram-positive aerobes, but may differ in their chemical characteristics, except for the menaquinone structure, which invariably contains eight or nine isoprene units. They are very common in soil, but some species are animal or plant pathogens (Goodfellow, 1992).

Nocardia of the family *Nocardiaceae*. Morphological traits include the presence of both vegetative and aerial hyphae, sometimes rudimentary, fragmenting into nonmotile elements. Chains of nonmotile spores may be formed. Cell wall components include meso-DAP, arabinose, and galactose. *Nocardiaceae* are mesophilic and grow on simple media containing ammonia, nitrates, or amino acids as nitrogen sources and glucose or acetate as carbon sources. Growth is slow; the doubling time is about 5 h, at least for the species reported. A variety of antibiotics are produced by *Nocardiaceae*; however, the strains producing the most important ones—rifamycins, vancomycin, and ristocetin—have recently been attributed to different genera, such as *Amycolatopsis* of the family *Pseudonocardiaceae*.

Amycolatopsis. This genus has recently been proposed to accommodate species formerly classified as *Norcardia*, which do not contain mycolic acids. The other taxonomic traits are similar to those of *Norcardia*, except for the presence of branched-chain fatty acids in the *Amycolatopsis* cell membrane. Several species have been described which produce antibiotics belonging to different biosynthetic classes. Glycopeptides are rather frequent, and among them are the therapeutically important vancomycin from *A. orientalis*, and ristocetin from *A. orientalis* subsp. *Lurida*. Other *A. orientalis* strains also produce an elfamycin antibiotic and the muraceins, which are muramyl peptide derivatives found to be inhibitors of angiotensin-converting enzymes (Lechervalier et al., 1988; Goodfellow, 1992; Sasaki et al., 1998). *A. mediterranei* is the producer of rifamycins, an important group of ansamycins. The original strain produces a complex of several components, among which rifamycin B was selected and used for the preparation of semi-synthetic derivatives. Rifamycin SV was first prepared chemically from rifamycin B, but it was also isolated from a *Nocardia* species.

Saccharopolyspora of the family *Pseudonocardiaceae*. Organisms of this genus, which includes only two validated species, differ from *Streptomyces* species in two respects: the tendency of their substrate mycelium to fragment, and the composition of the cell wall, which contains meso-DAP, galactose, and arabinose. The species of interest is *S. erythraea* (formerly *Streptomyces erythreus*), the producer of the well-known antibiotic erythromycin (Zhou et al., 1998).

1.1.2.3 The families *Streptosporangiaceae* and *Thermomonosporaceae*

Streptosporangineae, a suborder that contains the family *Streptosporangiaceae* and *Thermomonosporaceae*, are a group of rather diverse actinomycetes forming a substrate mycelium bearing aerial hyphae that differentiate into short chains of spores or into sporangia containing either motile or nonmotile spores. A characteristic trait is the presence of the sugar

maduraose in whole-cell hydrolysates; however, in some species this is present only in trace amounts. The cell wall contains meso-DAP and has no characteristic sugar. Antibiotics have been isolated from several strains belonging to genera of this group, such as *Streptosporangium* or *Planobispora*. However, *Actinomadura* is the only genus producing a large variety of secondary metabolites (Goodfellow, 1992).

Strains of the genus *Actinomadura* form colonies that are similar in appearance to those of *Streptomyces* species. However, they can be distinguished microscopically: in contrast to the genus Streptomyces, the spore chains are usually short and the spore diameter noticeably exceeds that of the hyphae. Actinomadurae are rather slow-growing organisms: formation of the spore-bearing aerial mycelium requires 10 - 14 days under optimal conditions. Their natural habitat is soil. Two species are pathogenic for man, causing actinomycetomas by infecting open wounds. Little is known about the physiology or the genetics of Actinomadurae. Conditions for growth are similar to those of other actinomycetes. Sugars and inorganic salts are normally sufficient as nutrients; starch and proteins are hydrolyzed and can sustain growth. Unlike Micromonosporaceae, chitin and xylan are not utilized (Venugopal and Venugopal, 1990; Tomita et al., 1991).

About 350 antibiotics have been isolated from *Actinomadura* strains (Table 1). Most frequently found are the ionophoric polyethers, such as the madurimicins produced by *A. yunaensis* and cationomycin produced by *A. azurea.* Also frequent are the anti-tumor anthracyclines, prominent among which are the carminomycins produced by *A. roseoviolacea* and other strains. Peptides of interest include the dalbaheptides parvodicin (*A. parvosata*), A-40926 (*Actinomadura* sp. ATCC 39727) and the angiotensin-converting enzyme inhibitor I-5B (*A. spiculosospora*). Characteristically, classical antibacterial macrolides are not produced; however, structurally similar products, the macrolactams, have been isolated from *A. fulva* and other strains (Lechervalier et al., 1988).

1.2 *Kitasatospora setae* and its completed genome sequence

The genus *Kitasatospora* is morphologically similar to that of *Streptomyces*. Typical characteristics of *Kitasatospora* are the production of spores and mycelia in a submerged culture, and the presence of both LL-and meso-diaminopimelic acids (DAP) in the cell wall (Omura et al., 1987) .

The genome sequence of *K. setae* was completely determined in 2010 by Ichikawa et al (Ichikawa et al., 2010). The genome is a single linear chromosome of 8,783,278 bp with terminal inverted repeats (TIR) of 127,148 bp, and is predicted to encode 7,569 protein-coding genes, 9 rRNA operons, 1 tmRNA and 74 tRNA genes. The average GC content of the chromosome was 74.2%, which is among the highest in actinobacteria. No critical difference was found between *K. setae* and *Streptomyces* species in the predicted primary metabolism, including carbohydrate metabolism, amino acid metabolism, nucleic acid metabolism and respiration. About 34% of *K. setae* ORFs had orthologs in all four *Streptomyces* genomes, i.e., *S. coelicolor*, *S. avermitilis*, *S. griseus* and *S. scabies*.

Regarding genes related to developmental regulation, classical differentiation genes in *Streptomyces* were found to have orthologs in *K. setae*: for example, *bld* genes, whose mutations in *S. coelicolor* cause defects in aerial growth; and *whi* genes which are involved in sporulation. However, the *amf* genes which are necessary for the synthesis of the AmfS surfactant protein do not exist in the *K. setae* genome. The initiation of secondary metabolite synthesis is known to be linked with morphological differentiation in *S. griseus* via the γ-butyrolactone autoregulator cascade. In this regard, it is noteworthy that *K. setae* possesses three homologues of the autoregulator receptor: KsbA, KsbB (There are 2 identical copies of KsbB because their genes are located in the TIR.) and KsbC. However, KsbA has been shown to be involved only in secondary metabolism, but not in morphological differentiation,

whereas the involvement of the other two homologues remains to be clarified. In addition, the AfsA family protein has been reported to be a crucial enzyme to synthesize the γ-butyrolactone autoregulator in *Streptomyces*. Interestingly, three AfsA family proteins were encoded on the genome of *K. setae*: KsbS2, (There are 2 identical copies of KsbS2 because their genes are located in the TIR.), KsbS3 and KsbS4. These findings suggested that there might be a more complicated signaling network for secondary metabolism and/or morphological differentiation in *K. setae*.

Bacteria belonging to the genus *Kitasatospora* have been explored as potential new sources of various bioactive metabolites. *K. setae* is known to produce setamycin (bafilomycin B1 (**1**)) (Bowman et al., 1988; Otoguro et al., 1988), a macrolide antibiotic which specifically inhibits the V-ATPase, controlling pH in lysosomes. In addition to setamycin, 24 genes or gene clusters in the genome of *K. setae* are predicted to be involved in the biosynthesis of secondary metabolites (Ichikawa et al., 2010). Of the 24 clusters, 5 are estimated to play a role in terpene biosynthesis, 12 in the synthesis of polyketides or non-ribosomal peptides, 2 in siderophore synthesis and 5 in the synthesis of lantibiotics and others.

1.3 γ-Butyrolactone autoregulators and their receptors in actinomycetes

Antibiotic production in the actinomycetes generally proceeds in a growth phase-dependent manner. In liquid culture, it begins when the culture enters stationary phase. In agar-grown cultures, it coincides with the onset of morphological differentiation. The occurrence of mutants deficient in both antibiotic production and the formation of aerial hyphae indicate at least some common elements of genetic control (Chater and Bibb, 1997). Most antibiotics are the products of a complex biosynthetic pathway with a cluster of genes (generally 20 - 30) dedicated to the synthesis of any one compound. These gene clusters

usually contain pathway-specific regulatory genes which act as transcriptional activators, which occasionally are controlled 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, diffusible γ-butyrolactone signaling molecules (Sato et al., 1989; Yamada and Nihira, 1998), imbalances in metabolism (Hood et al., 1992) and various physiological stresses (Hobbs et al., 1992; Yang et al., 1995). In addition to these positive effectors of antibiotic production, antibiotic synthesis may also be subject to metabolite repression and/or inhibition by readily utilizable sources of nitrogen (generally NH_4^+), phosphate and/or glucose (Demain, 1992; Demain and Fang, 1995; Chater and Bibb, 1997). The various factors that influence antibiotic production in actinomycetes are not readily accommodated in a simple unifying model. However, it seems reasonable to propose an overall regulatory influence of 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.

γ-Butyrolactones are produced by many, if not all streptomycetes (Horinouchi and Beppu, 1992; Yamada, 1999), and by other genera of actinomycetes (Choi et al., 2003, 2004). They are implicated in the onset of secondary metabolism, as well as, in some strains, that of morphological differentiation (Table 2). The structure of fourteen 2,3-disubstituted γ-butyrolactones that are from seven *Streptomyces* species (Fig. 1.3) have been determined so far, and they differ in length, branching, and the stereochemistry of their fatty acid side-chains (Yamada, 1999; Horinouchi, 2002; Nihira, 2002). The effectiveness of these autoregulators which are active at nanomolar concentrations, as well as the presence of specific receptor proteins as mediators of autoregulator signaling implies that these γ-butyrolactone autoregulators would be regarded as *Streptomyces* hormones. The γ-

butyrolactones bind to cytoplasmic receptor proteins and inhibit their binding to specific DNA targets as dimers. Most of these receptor proteins act as repressors, so that binding to γ-butyrolactone induces expression of the target genes. The sections below summarize the autoregulator-receptor pairs whose regulatory effects on secondary metabolism and/or morphological differentiation have been determined thus far, followed by other receptor homologues that play important roles in actinomycetes.

1.3.1 A-factor and ArpA

An autoregulator A-factor [2-(6-methyheptanoyl)-3*R*-hydroxymethy-4-butanolide], which triggers the production of streptomycin and aerial mycelium formation in *S. griseus* (Fig. 1.3), was originally discovered by Khokhlov and coworkers in Russia (Khokhlov, 1980).

This A-factor acts as a diffusible extracellular molecule to recover wild-type phenotypes in streptomycin and in the spore-deficient mutant *S. griseus* HH1.

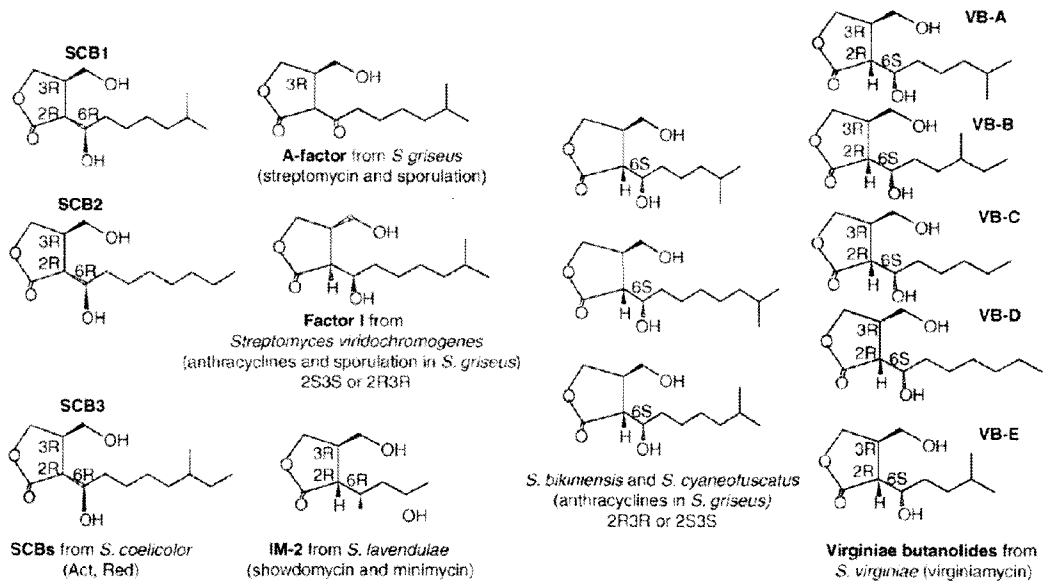

Fig. 1.3 Chemical structures of γ-butyrolactones from *Streptomyces*. The name of the signaling molecules appear in bold, and the antibiotic that these molecules effect or its other functions are shown in brackets.

The A-factor receptor protein was identified and purified in the cytoplasm of *S. griseus* with a radioactive ligand (Miyake et al., 1990), and its gene (*arpA*) was cloned by collaboration between two research groups (Onaka et al., 1995). The resulting gene product, ArpA, forms a homodimer. The number of receptor molecules (30-40 per genome) is very low and its K_d value has been estimated to be 0.7 nM.

The A-factor-independent streptomycin producer, *S. griseus* 2247, was found to be a receptor-deficient strain (Miyake et al., 1990). The finding of this mutant has inspired the screening for similar mutants. The characterization of these mutants indicated that the A-factor receptor was pleiotropic in nature, able to down-regulate the production of streptomycin and morphogenesis with repression in the absence of restored A-factor. Intact ArpA acts as a transcriptional repressor of the *adpA* gene (A-factor-responsive transcriptional activator) controlling the onset of sporulation and secondary metabolism. When A-factor accumulates to a concentration sufficient to combine with the receptor ArpA, this binding induces the release of ArpA from the promoter of *adpA*, triggering the expression of the *adpA* gene. The AdpA protein activates the transcription of *strR* (the pathway-specific transcriptional activator); subsequently, StrR activates transcription of the streptomycin biosynthetic gene cluster (Onaka et al., 1995; Onaka and Horinouchi, 1997). By repressing the *adpA* gene, ArpA indirectly represses transcription of the *adsA* gene encoding an extracytoplasmic sigma factor necessary for morphological differentiation (Yamazaki et al., 2000). However, it is not clear at present whether ArpA participates in the regulation of A-factor production in *S. griseus*.

1.3.2 Virginiae Butanolide (VB) and BarA

Virginiae butanolides (VBs) composed of 5 congeners (VB-A~E), each of which triggers the production of virginiamycin M_1 and S in *S. virginiae* (Fig. 1.3), were first found as a

signal substance or as signal substances named IM (inducing material) by Yanagimoto and coworkers (Yanagimoto and Terui, 1971). When VB was produced at 12 hours of cultivation in the late exponential growth phase, virginiamycin production was initiated 2 hours later.

Since VBs are secreted into the culture broth, triggering virginiamycin production in other cells, there should be a receptor protein that mediates the signal to facilitate the transcription of virginiamycin biosynthetic genes. In a series of experiments designed to examine this hypothesis, the VB receptor protein (BarA) was identified and purified with [^{3}H]-labeled VB-C_7 (Nihira et al., 1988; Kim et al., 1989, 1990) and the gene encoding BarA (*barA*) was cloned and characterized (Okamoto et al., 1995). BarA forms a homodimer with an M.W. of 52 kDa. The number of receptor molecules (30-40 per genome) is very low and its K_d value toward VBs has been shown to be 1.1 nM.

BarA possesses a helix-turn-helix (HTH) DNA-binding motif on its N-terminus, strongly suggesting that BarA is a regulatory protein capable of binding to DNA. *In vitro* (Kinoshita et al., 1997, 1999) and *in vivo* (Kinoshita et al., 1997; Nakano et al., 1998, 2000) analyses have demonstrated that BarA in the absence of VB works as a DNA-binding transcriptional repressor and that the release of BarA in the presence of VB from the promoter region of its target gene(s) results in virginiamycin (VM) production. One of the target genes is *barB*, located 383 bp away from the *barA* gene, which is transcribed in the same direction as *barA*. The binding activities of BarA to specific DNA fragments (BAREs) in the *barB*- and *barA*-promoter regions have been confirmed by the SPR (surface plasmon resonance) technique (Kinoshita et al., 1997, 1999). With respect to the existence of an HTH motif on its N-terminus, BarB also seems to be a DNA-binding regulatory protein, although the actual function of BarB remains unclear (Matsuno et al., 2004).

1.3.3 IM-2 and FarA

In comparison with A-factor and VB, IM-2 [(2*R*, 3*R*, 6*R*)-2-(hydroxybutyl)-3-3(hydroxy- methyl)-4-butanolide] is a unique autoregulator showing both positive and negative effects on secondary metabolism (Fig. 1.3). In the stationary phase, IM-2 switches the biosynthetic pattern of secondary metabolites from D-cycloserine to nucleoside-type antibiotics and also induces blue pigment production in *Streptomyces lavendulae* FRI-5.

The IM-2 binding protein (FarA) was purified (Ruengjitchatchawalya et al., 1995) and the corresponding gene (*farA*) was cloned (Waki et al., 1997). FarA also has an HTH motif on its N-terminus; this feature strongly suggests that FarA is also a DNA binding regulatory protein whose function is mediated in combination with IM-2. *In vitro* studies of FarA have indicated that FarA represses its own synthesis under the control of IM-2.

Functional analysis of FarA by gene disruption (Kitani et al., 2001) has indicated that the IM-2-FarA system has a unique signaling cascade, in which the presence of both IM-2 and intact FarA are necessary for the suppression of D-cycloserine biosynthesis. FarA is also confirmed to be necessary for the IM-2 biosynthesis.

1.3.4 SCB1 (*Streptomyces coelicolor* γ-butyrolactone autoregulators 1) and ScbR

Recently, SCB1 [(2*R*, 3*R*, 6*R*)-2-(6-methylheptanoyl)-3-(hydroxymethyl)-4-butanolide], which induces the precocious production of the blue-pigmented polyketide actinorhodin (Act) and the red-pigmented tri-pyrolle undecylprodigiosin (Red) at the early stationary phase (Fig. 1.3), was discovered in *S. coelicolor* A3(2), the most genetically characterized model actinomycete (Takano et al., 2000). Additionally, diverging genes involved in γ-butyrolactone synthesis (*scbA*) and γ-butyrolactone binding (*scbR*) were isolated (Takano et al., 2001). ScbR regulates the transcription of both *scbA* and itself by binding to the divergent

promoter region, and SCB1 inhibits the binding. *In vivo* analysis demonstrated that ScbR represses its own expression while activating the expression of *scbA*. In striking contrast to the A-factor model in *S. griseus*, deletion of *scbA* in *S. coelicolor* results in precocious overproduction of both Act and Red, whereas deletion of *scbR* results in markedly delayed Red synthesis with only a small delay in Act production. Furthermore, a specific interaction between ScbR and the *kasO* gene, a putative pathway-specific regulatory gene in the cryptic type I polyketide biosynthetic gene cluster, was observed (Takano, 2006), showing that a γ-butyrolactone-binding protein directly regulates a secondary metabolite pathway-specific regulatory gene in *S. coelicolor* A3(2).

1.3.5 Other autoregulator receptors in actinomycetes

The enormous increase in the number of γ-butyrolactone receptor homologues (some of which have been characterized in various *Streptomyces* species, as shown in Table 2) that have been identified reflects the fact that the nucleotide sequences of many gene clusters that encode production of secondary metabolites have recently become available. At least 22 out of 33 genes encoding homologues of γ-butyrolactone receptors are located close to antibiotic biosynthetic genes and/or have been shown to regulate antibiotic production. Thus, it seems that γ-butyrolactones are strongly associated with the regulation of antibiotic production and that most of the receptors are pathway-specific.

In many streptomycetes, multiple γ-butyrolactone receptor homologues are present in the genome, and in some strains, they are all located in antibiotic biosynthetic gene clusters. A particularly interesting example is the tylosin biosynthetic gene cluster of *Streptomyces fradiae*, which contains, remarkably, no fewer than five putative regulatory genes (Bate et al., 1999): *tylP* (a typical receptor homologue) and *tylQ* (encoding pseudo-receptor protein homologue), *tylT* and *tylS* (encoding *Streptomyces* antibiotic regulatory protein (SARP)

Table 2 γ-Butyrolactone signalling proteins identified in actinomycetes (Willey & Gaskell, 2011)

Species	γ-Butyrolactone autoregulator	Biosynthetic enzyme	Receptor	Regulated antibiotic	Morphogenesis regulated?	References
S. griseus	A-factor	AfsA	ArpA	Streptomycin	Yes	(Horinouchi, 2007)
S. coelicolor	SCB1, 2, 3, Acl-1	ScbA	ScbR	Act, Red, *kas*	No	(Hsiao *et al.*, 2009; Joo *et al.*, 2007; Takano *et al.*, 2001, 2005)
S. virginiae	VB-A to E	BarX	BarA, BarB	Virginiamycin	No	(Nakano *et al.*, 1998)
S. lavendulae	IM-2	FarX	FarA	Showdomycin Minimycin	No	(Kitani *et al.*, 1999)
S. viridochromogenes	Factor I			Anthracyclins	Yes	(Grafe *et al.*, 1982)
S. bikiniensis	3, un-named					(Takano, 2006)
S. fradiae			TylP, TylQ	Tylosin	No	(Bignell *et al.*, 2007)
S. venezuelae		JadW1	JadR2	Jadomycin B	Yes, conditional	(Wang & Vining, 2003)
S. natalensis		Orf2	SngR	Natamycin	Yes	(Lee *et al.*, 2005)
S. acidiscabies		SabA	SabS, SapR	Polyketide WS5995B	Yes, conditional	(Healy *et al.*, 2009)

Table 2 (continued)

Species	γ-Butyrolactone autoregulator	Biosynthetic enzyme	Receptor	Regulated antibiotic	Morphogenesis regulated?	References
S. rochei		SrrX	SrrA, SrrB, SrrC	Lankamycin, Lankacidin	Yes	(Arakawa *et al.*, 2007)
S. pristinaespiralis			SpbR	Pristinamycin	Yes	(Folcher *et al.*, 2001)
S. tendae			TarA	Nikkomycin	No	(Engel *et al.*, 2001)
S. clavuligerus			BrpC	Cephamycin, Clavulanic acid	No	(Kim *et al.*, 2004)
S. ansochromogenes			SabR	Nikkomycin	Yes, conditional	(Li *et al.*, 2003)
S. ambofaciens			AlpZ	Alpomycin	No	(Bunet *et al.*, 2008)
K. setae			KsbA	Bafilomycin	No	(Choi *et al.*, 2004)

homologues), and *tylR*. Four of these genes (i.e., all except *tylT*, which is not involved in the regulation of tylosin production) appear to encode a regulatory cascade in which TylP, in the absence of an unidentified γ-butyrolactone, represses *tylS* and *tylQ* transcription. *tylQ*, in turn, represses transcription of *tylR*, which functions as an activator of tylosin biosynthetic genes. Interestingly, all three of these proteins, which have yet to be shown to bind any ligand, act as negative regulators of antibiotic production in pathway-specific regulatory cascades. An additional example is the γ-butyrolactone-autoregulator-receptor system involved in lankacidin and lankamycin production and morphological differentiation in *Streptomyces rochei* (Arakawa et al., 2007). This system involves three large linear plasmids (pLA2-L, -M and -S), and on the largest one, pLA2-L, three receptor homologues (*srrA*, *srrB* and *srrC*) are present with the biosynthetic gene clusters of lankacidin, lankamycin, an unknown type-II polyketide compound and carotenoid. The disruption of *srrA*, encoding a typical autoregulator homologue, did not affect the production of two antibiotic productions but showed no sporulation. However, in the case of *srrB* and *srrC* (two pseudo-receptor homologues), *srrC* disruption showed the same effect as *srrA*, whereas the *srrB* disruptant showed an increased production of both lankacidin and lankamycin, while spore formation was slightly delayed. The presence of multiple receptors that repress or activate different regulatory genes with a different function could generate complex functions that might operate at different hierarchical levels.

1.4 β-Carboline alkaloids: their pharmacological activities and biosynthesis

β-Carboline alkaloids are a large group of natural and synthetic indole alkaloids with different degrees of aromaticity, some of which are widely distributed in nature, including various plants, marine creatures, insects, and mammalians, as well as human tissues and body

fluids. Among over 200,000 natural products in the Dictionary of Natural Products database (on DVD), 606 substances contain β-carboline in their structures. Figure 1.4 shows the proportion of β-carboline alkaloids which are produced in different organisms. One-third of β-carboline alkaloids are from plants, mostly flowering plants, while about 25% are produced from sea animals such as tunicates and sponges. The other 5% of these chemicals are from bacteria in the groups actinomycetes, fungi, algae, cyanobacteria and yeast.

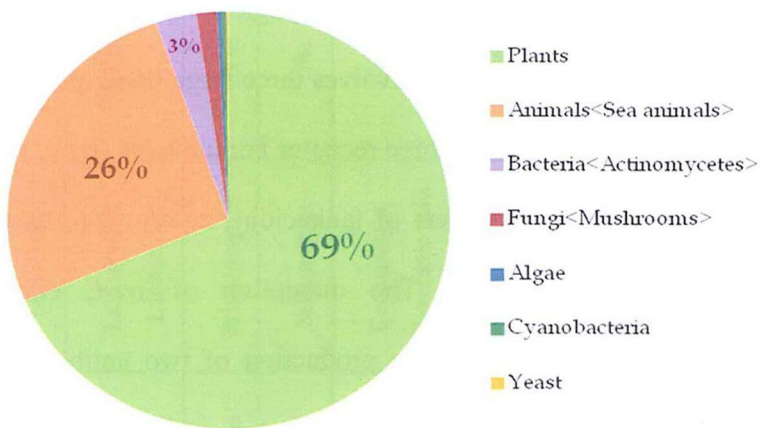

Fig 1.4 Pie chart illustrating the proportions of β-carboline alkaloids produced from various organisms.

When the pharmacological properties of the β-carboline alkaloids were characterized, these chemicals demonstrated a broad spectrum of properties, including sedative, anxiolytic, hypnotic, anticonvulsant, antitumor, antiviral, antiparasitic and antimicrobial activities. Previously, numerous reports investigated the effects of β-carboline alkaloids on the central nervous system, since these compounds were widely used as hallucinogenic drinks or snuffs. However, recent interest in these alkaloids has been focused on their potent antitumor, antiviral, antimicrobial and antiparasitic activities. In the investigation of antitumor activities of numerous β-carboline derivatives bearing various substituents at different positions of the β-carboline nucleus, the structure-activity relationships of β-carboline derivatives have been

reported (Cao et al., 2007). These reports indicate that (1) the β-carboline structure is an important basis for the design and synthesis of new antitumor drugs; (2) appropriate substituents at positions 1, 3 and 9 of the β-carboline ring might play a crucial role in determining their enhanced antitumor activities; (3) the antitumor potencies of β-carboline derivatives were enhanced by the introduction of a benzyl substituent into position 2; (4) the acute toxicity of β-carboline derivatives was reduced dramatically by the introduction of an appropriate substituent into positions 3 and 9; (5) the β-carboline derivatives have the potential to be used as antitumor drug leads.

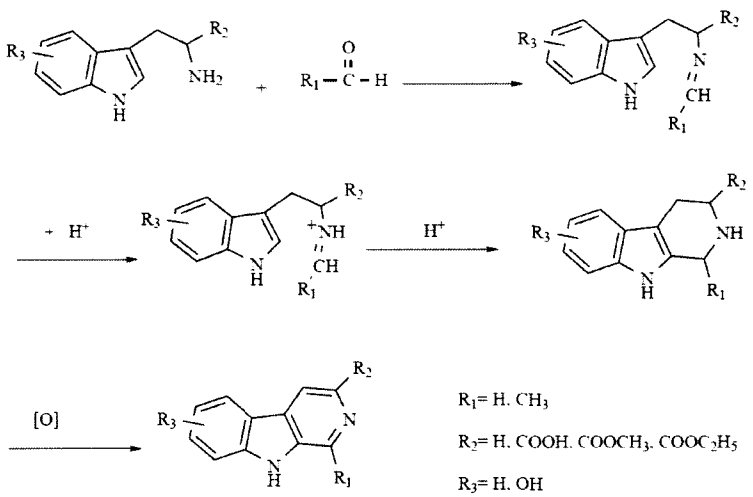

Fig. 1.5 Pictet-Spengler condensation (Cao et al., 2007) between indoleamines and acetaldehyde or formaldehyde to give simple tetrahydro-β-carboline (THβCs) alkaloids. Oxidation of THβCs provides β-carbolines.

Regarding the synthesis of β-carboline alkaloids, it is well known that the simple β-carboline alkaloids, such as tetrahydro-β-carboline-3-carboxylic acid, are easily formed from tryptophan or tryptamine and formaldehyde or pyruvate or acetate precursors by a known reaction, the Pictet-Spengler reaction (Fig. 1.5) (Cao et al., 2007). The Pictet-Spengler reaction yields β-carboline and tetrahydroquinoline structures essential to the biosynthesis of

thousands of natural plant products. Enzymes that catalyze this reaction have been isolated from several plant alkaloid biosynthetic pathways. Strictosidine synthase (Kutchan et al., 1988), one "Pictet-Spenglerase", is the central enzyme in the biosynthesis of the monoterpene indole alkaloids, including vinblastine, yohimbine, ajmaline, camptothecin, and strychnine, all of which are found in a wide variety of plant species. Strictosidine synthase diastereoselectively converts the substrates tryptamine 1a and secologanin to the β-carboline product strictosidine (Fig. 1.6) (Maresh et al., 2008). Strictosidine serves as the biosynthetic precursor to all terpene indole alkaloids.

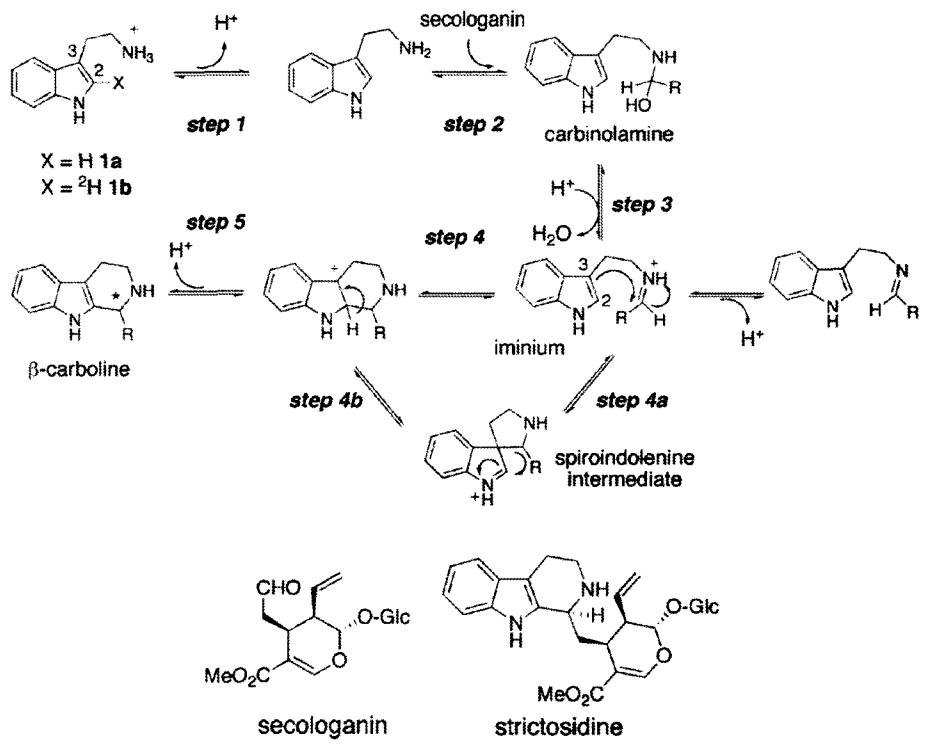

Fig. 1.6 Pictet-Spengler reaction mechanism (Maresh et al., 2008).

The Pictet-Spengler reaction is essentially a two-part reaction. First, an electro-rich aromatic amine and an aldehyde condense to form an iminium species (Fig 1.6, steps 1-3). Second, an electrophilic aromatic substitution reaction occurs in which the aryl amine attacks the electrophilic iminium to yield a positively charged intermediate (Fig. 1.6, step 4) which is then deprotonated to yield the β-carboline product(s). In nonenzymatically catalyzed

reactions, two enantiomers are typically formed, but strictosidine synthase catalyzed the asymmetric synthesis of the strictosidine diastereomer (asterisk in Fig. 1.6). Notably, both carbons 2 and 3 of tryptamine are nucleophilic (Fig. 1.6). Therefore, after iminium formation, Pictet-Spengler reactions that utilize indole amine substrates can proceed either by attack of carbon 2 to directly yield the six-membered ring intermediate (Fig. 1.6, step 4) or by attack of carbon 3 to yield a spiroindolenine intermediate (Fig. 1.6, step 4a) that would then undergo a 1,2-alkyl shift (Fig. 1.6, step 4b) to form the product. Evidence for both mechanisms in solution exists, and the predominant mechanism is not entirely clear. The mechanism for the enzymatic reaction is not known.

1.5 Overview and objectives of this study

The genome sequence of *K. setae* reveals two more homologues of the autoregulator receptors other than KsbA, a negative regulator of bafilomycin production. Despite of high similarity to known receptor such as FarA and BarA from *Streptomyces*, one homologue (KsbC) shows a lack of an important residue for autoregulator recognition. Moreover, gene organization of KsbC comparatively resembles to autoregulatory island in *Streptomyces virginiae*, but KsbC is not located in or near any putative biosynthetic gene cluster. All intriguing characteristics suggested that KsbC might play an important role, whether similar to KsbA or not, on the morphological and/or physiological differentiation in *K. setae*. Therefore, we attempted to characterize the regulatory function of KsbC by gene disruption. All phenotypic analyses of the *ksbC* disruptant are described in Chapter 2. In addition, the *ksbC* mutant showed precocious and abundance of unknown metabolite production. Thus, we purified and elucidated the structure of the increased compounds and discovered three novel derivatives of β-carboline alkaloids for the first time in *K. setae*. The full details of these experiments and characterizations are given in Chapter 3.

Chapter 2

Pleiotropic control of secondary metabolism and morphological development by KsbC, a butyrolactone-autoregulator receptor homologue in *Kitasatospora setae*

2.1 Introduction

Members of the genus *Streptomyces* have been extensively studied due to their complex developmental life cycle and their ability to synthesize a vast array of important secondary metabolites used in human/veterinary medicine and agriculture. Recently, the rapid accumulation of genome information has enabled elucidation of the physiological mechanisms at the molecular level, in addition to pointing the hitherto undiscovered ability to produce novel secondary metabolites, which are a promising source of new clinically useful compounds. Actinomycetes other than those in the genus *Streptomyces* are often called non-*Streptomyces* actinomycetes: these include actinomycetes of the genera *Kitasatospora*, *Micromonospora*, *Actinoplanes*, *Amycolatopsis*, and *Nocardia*, all of which produce useful natural compounds in the manner of the *Streptomyces* species (Table 1). The genus *Kitasatospora,* members of which exhibit lifestyles and morphological development similar to those of *Streptomyces* species, is phylogenetically close to the genus *Streptomyces*, and belongs to the same family *Streptomycetaceae*. *Kitasatospora setae* NBRC 14216^T produces bafilomycins A1 and B1 (**1**), specific inhibitors of vacuolar H^+-ATPase commonly used as biochemical reagents to investigate molecular transport in eukaryotic cells (Fig. 2.1A) (Bowman et al., 1988; Otoguro et al., 1988). The complete genome sequence revealed that *K. setae* has at least 24 genes or gene clusters for the biosynthesis of secondary metabolites, including bafilomycin (Ichikawa et al., 2010). A vast majority of these genes and clusters

play unknown roles in the biosynthetic processes and are presumably cryptic biosynthetic pathways. An improved understanding of the systems for regulating secondary

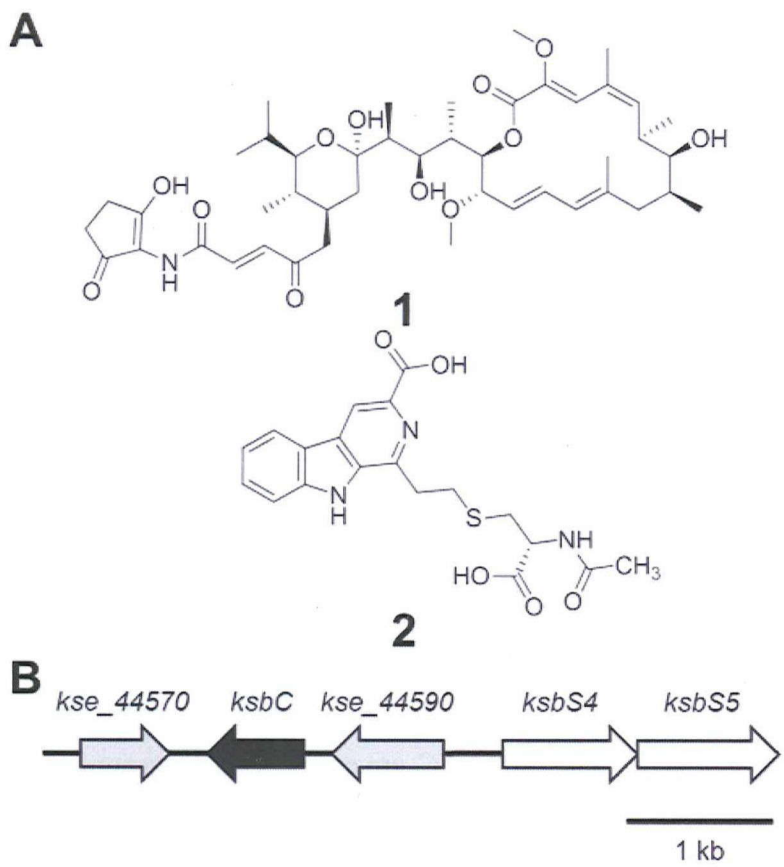

Fig. 2.1 Chemical structures of metabolites of *K. setae* (A) and organization of the ksbC locus in *K.setae* (B). (A) Structures of bafilomycin B1 (**1**) and kitasetaline (**2**). (B) Grey arrows indicate putative regulatory genes, and white arrows indicate putative biosynthetic genes for a KsbC ligand.

metabolism in *K. setae* not only might reveal common features and differences of the genetic information between the genera *Kitasatospora* and *Streptomyces*, but also could provide a great opportunity to discover novel natural compounds.

γ-Butyrolactone-autoregulator signaling cascades are known to be the major regulatory systems for *Streptomyces* secondary metabolism (Bibb, 2005; Takano, 2006), and consist of an autoregulator synthase (AfsA-family proteins), a cognate receptor protein, and receptor-

controlled target proteins, encoded by genes that are frequently clustered at the same locus. In this system, the autoregulator receptor binds to a specific DNA sequence called an autoregulatory element (ARE) in the promoter region of its target genes, repressing their transcription (Folcher et al., 2001). Binding of the autoregulator prevents the receptor from interacting with DNA, allowing transcription of the target genes, and in turn activating the coordinated expression of regulatory and enzymatic genes involved in secondary metabolism, and sometimes in morphological development. KsbA is an autoregulator receptor of *K. setae* that is the only receptor identified in non-*Streptomyces* actinomycetes by the conventional method using degenerate PCR primers (Choi et al., 2004). KsbA functions as a negative regulator of bafilomycin production but has no influence on morphological differentiation. The DNA-binding activity of KsbA and the target genes remain to be elucidated, and the adjacent regions of the *ksbA* gene have no plausible *afsA*-family genes. Searches of the genome sequence of *K. setae* demonstrated that, in addition to *ksbA*, two more putative autoregulator receptor genes, *ksbB* and *ksbC*, are encoded on the genome, together with *afsA*-family genes in the proximal region, and thus may be involved in the regulation of secondary metabolism (Ichikawa et al., 2010). The genomes of well-studied *Streptomyces* strains such as *S. coelicolor* A3(2), *S. griseus*, and *S. avermitilis*, have only one copy of *afsA*-family genes. These findings prompted us to investigate the function of additional autoregulator receptors in *K. setae*, which might form a more complicated signaling network for secondary metabolism and/or morphological development compared with that of *Streptomyces* species.

In this chapter, we report the role of KsbC in the regulation of secondary metabolism and morphological development, and demonstrate that KsbC positively controls bafilomycin production and aerial mycelium formation. Moreover, the *ksbC* mutant showed precocious and abundant production of the metabolite, a novel β-carboline alkaloid named kitasetaline.

(The purification and structure elucidation of kitasetaline as well as identification of its biosynthetic genes are described in Chapter 3.)

2.2 Materials and methods

2.2.1 Bacterial strains, plasmids, and growth conditions

K. setae NBRC 14216^T from the NITE Biological Resource Center (NBRC), Japan, was grown on ISP medium 2 (Becton Dickinson and Company, Franklin Lakes, NJ, USA). *Escherichia coli* DH5α was used for general DNA manipulation, the DNA methylation-deficient *E. coli* strain ET12567 containing pUZ8002 (Paget et al., 1999) was used for *E. coli/Kitasatospora* conjugation. *E. coli* BL21(DE3) pLysS and the plasmid pET-15b, which were used for the expression of recombinant KsbC (rKsbC), were obtained from Novagen. The plasmids used were pUC19 for general cloning, and pKU451, pKU474 and pKU250 for gene disruption (Komatsu et al., 2010; Miyamoto et al., 2011). For complementation, pENTR (Invitrogen) was used for DNA cloning, and pLT113 (Miyamoto et al., 2011) was used to introduce DNA into *Streptomyces*. The media conditions, antibiotic concentrations, and general *E. coli* and *Kitasatospora/Streptomyces* manipulations were as described previously (Kieser et al., 2000) Spores (1 x 10^8 CFU) of *K. setae* strains were inoculated into 70 ml YMM medium in a 500 ml baffled flask, and mycelia were harvested after 36 h of cultivation at 28°C. The mycelia were washed, resuspended in fresh YMM medium (Aroonsri et al., 2012a) and stored at -80°C until use as a seed culture. All the primers are listed in Table 3.

2.2.2 Disruption of the *K. setae ksbC* gene

A 2.0 kb *ksbC*-upstream fragment was amplified using the primer pair ksbC-up-Fw and ksbC-up-Re, and a 2.2 kb *ksbC*-downstream fragment was amplified using the primer pair

Table 3 Oligonucleotides used in this study

Primer	Sequence (5'-3')*
For construction of ksbCdisruptant and ksbC-complemented strain	
ksbC-up-Fw	CCCAAGCTTTGCGCGCCGAGCACCACGTTCT
ksbC-down-Fw	GGAATTCCCCGGCATGATCTGCAACCTG
ksbC-up-Re	GGAATTCGCGTTCCTGCTTCGCCAT
ksbC-down-Re	CCAAGCTTCGAAGTTGACGGTCGCCAGC
aphII-E-Fw	ATGGATTGCACGCAGGTTCTCC
aphII-E-Fw	ATGGATTGCACGCAGGTTCTCC
aphII-E-Re	GCGGCGATACCGTAAAGCACGA
aphII-E-Re	GCGGCGATACCGTAAAGCACGA
ksbC-comp-Fw	CACCGATTCCGCCCGGTGCGGACCTCCCG
ksbC-comp-Re	GGAGATGCTGGAACAGCTCCACGGCC
For overexpression of recombinant KsbC protein	
rKsbC-Fw	GGAATTCCATATGGCGAAACAGGAACGCGGCAC
rKsbC-Re	GGGGATCCCGTCAGTCGACGGCGGGGG
For quantitative RT-PCR analysis	
ksbA-qF	CAACTGCTGGTCAGCTCCTACAG
ksbA-qRe	GGGAGCAGGTAGGTCCACATC
ksbS4-qFw	AGCACCCGATCTTCTTCGACCA
ksbS4-qRe	GTAGGAGTGGAAGGAGCTGTCC
For gel-shift assay	
E1-Fw	CCCGGTCCGGACGGTGTCG
E1-Re	GATGTGGATGCTATGCACCGATTTGG
E2-Fw	CGCACATCGGGTCGTCGCAG
E2-Re	CTCGGCCCGCCACCTCGC
E3-Fw	CGTCGCCGGGCGTGTCGA
E3-Re	GATACAGACCGACCTGTCGGG

* Restriction sites are underlined.

ksbC-down-Fw and ksbC-down-Re. The fidelity of the amplified regions was confirmed by sequencing. The two resultant fragments were digested with *Eco*RI and *Hin*dIII, and cloned together into the *Hin*dIII site of pKU451. The resultant plasmid was cleaved by *Eco*RI and ligated with a kanamycin-resistance gene amplified by the primer pair aphII-E-Fw/aphII-E-Re using pKU474 as a template to yield pLT430. A 5.6 kb *Hin*dIII fragment, recovered from pLT430, was inserted into the *Hin*dIII site of pKU250 to generate pLT433 for *ksbC* disruption. *E. coli* ET12567 (pUZ8002) harboring pLT433 was conjugated with *K. setae*, and the wild-type gene was replaced with the disrupted allele (Δ*ksbC*) by homologous recombination. The genotype of the Δ*ksbC* candidates resistant to kanamycin and sensitive to thiostrepton was confirmed by PCR analysis.

2.2.3 Complementation of the *ksbC*-deletion mutant

The entire *ksbC* gene with its 504-bp upstream region was amplified by the primer pair ksbC-comp-Fw/ksbC-comp-Re, and then cloned into a pENTR vector to generate an entry clone. The entry clone was used with pLT113 in an LR reaction (LR Clonase Enzyme Mix; Invitrogen), resulting in pLT436. By intergenic conjugation and integration, the plasmid pLT436 was introduced into the *ksbC*-deletion mutant. The correct integration in the exconjugants was confirmed by PCR.

2.2.4 Analysis of *K. setae* secondary metabolites

The seed culture was spread onto 2.5 ml ISP medium 4 (Becton Dickinson and Company, Franklin Lakes, NJ, USA), and incubated at 28°C for 5 days. The agar culture was diced and extracted with 2 volumes of methanol. The methanol-extract was collected by centrifugation, and analyzed by the HPLC systems on an Inertsil ODS-3 column (4 μm; 4.6 x 250 mm; GL Sciences, Tokyo, Japan), using 80% CH_3CN containing 0.1% trifluoroacetic acid (TFA) as a mobile phase and detection at 245 nm for bafilomycin production, and with a

linear gradient system [eluents: H_2O containing 0.075% TFA (A), methanol containing 0.01% TFA (B); gradient 0-5 min 10% B, 5-55 min 10% B to 100% B; flow rate 1 ml/min; UV detection at 276 nm] for kitasetaline production. Commercial bafilomycin A1 and bafilomycin B1 (Fluka, USA), and purified bafilomycin C1 were used as standards for HPLC analysis.

2.2.5 Transcriptional analysis by semi-quantitative RT-PCR and quantitative RT-PCR

Total RNA was prepared from mycelia on ISP medium 4 with cultivation for 5 days using an RNeasy Mini kit (Qiagen), and treated with DNaseI (Takara Bio). The cDNA was synthesized using Superscript III RNase H^- Reverse Transcriptase (Invitrogen) and Random primers (Invitrogen) according to the manufacturer's instructions. For semi-quantitative RT-PCR, the PCR amplification was performed with GoTaq Green Master Mix (Promega KK) under the following conditions: 98°C for 3 min, followed by 35 cycles of 98°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The absence of DNA contamination was confirmed by RT-PCR without reverse transcriptase. Quantitative RT-PCR (qRT-PCR) was performed using the Applied Biosystems 7300 Real-Time PCR System and SYBR Green PCR Master Mix (Applied Biosystems) according to the supplier's recommendations. The reaction conditions were as follows: 95°C for 10 min, followed by 40 cycles of 15 s at 98°C for denaturation, and 1 min at 60°C for annealing and extension. A final dissociation stage was run to generate a melting curve and consequently verify the specificity of the amplification products. Gene expression was measured in triplicate and normalized to the mRNA level of the *hrdB* gene (*kse_54060*) using the relative standard curve method.

2.2.6 Overexpression and purification of rKsbC

The *ksbC* gene was amplified by PCR using genomic DNA of *K. setae* as a template and

the primer pair rKsbC-Fw/rKsbC-Re. A 657 bp *ksbC*-containing DNA fragment was digested with *Nde*I and *Bam*HI and then cloned in pET-15b digested with the same enzymes, resulting in pLT426, which was verified by sequencing. An LB culture of *E. coli* BL21(DE3) pLysS harboring pLT426 containing ampicillin (50 μg/ml) and chloramphenicol (25 μg /ml) was grown at 37°C, until the optical density at 600 nm reached 0.6, at which time 1 mM IPTG was added for induction. After an additional 3 h of cultivation, the cells were harvested by centrifugation, resuspended in buffer A [50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM *p*-(amidinophenyl)methanesulfonyl fluoride hydrochloride], and then disrupted by sonication. After centrifugation (6,230 *g*, 15 min, 4°C), the supernatant was directly applied to a His Microspin Purification Module (GE Healthcare Bio-Sciences). Proteins containing rKsbC were eluted with 500 mM imidazole (in buffer A) following the manufacture's recommendations. The concentration of protein was measured with a Bio-Rad protein assay kit using bovine plasma gamma globulin as a standard. The purity of rKsbC was analyzed by SDS-PAGE.

2.2.7 Gel-shift assay

The fragments E-1, E-2, and E-3 were amplified by PCR with the primer pairs E1-Fw/E1-Re, E2-Fw/E2-Re, and E3-Fw/E3-Re, respectively, and cloned into the *Hinc*II site of pUC19. The DNA probes were labeled by PCR using these plasmids as templates with an FITC-labeled M13-47 primer and RV primer. After FITC-labeled probe (7.5 ng) and rKsbC (1 μg) were mixed and incubated at 25°C for 10 min, the reaction mixture was separated at 4°C by electrophoresis. The conditions used for the detection of DNA retardation were as previously described (Kitani et al., 2008), and labeled DNA fragments were detected using an FMBIO II Multi-View (Hitachi Software Engineering) or Typhoon 9210 Variable Mode Imager (GE Healthcare).

2.3 Results

2.3.1 Bioinformatic analysis of autoregulator-receptor homologues of *K. setae*

In the genome sequence of *K. setae* NBRC 14216^T, we previously found two putative butyrolactone-autoregulator receptor genes, *ksbB* (*kse_01050t* and *kse_75690t*; identical genes encoded in the left and right terminal inverted repeats) and *ksbC* (*kse_44580*), in addition to *ksbA*, which acts as a negative regulator of bafilomycin production. A phylogenetic tree of KsbB and KsbC with other autoregulator receptors, including KsbA and pseudo-receptor regulators in *Streptomyces* (Fig. 2.2A), indicated that the KsbA/B/C proteins are grouped in the same branch, but are positioned at the outmost clade of the *Streptomyces* autoregulator receptors, and do not belong to the clade of pseudo-receptor regulators in *Streptomyces* species. The two groups (autoregulator receptors and pseudo-receptor regulators) can be distinguished easily by their p*I* values: autoregulator receptors have p*I* values of around 5 (p*I* of 5.1 for ArpA (Onaka et al., 1995) and 5.1 for BarA (Okamoto et al., 1995)), whereas pseudo-receptor regulators show very basic p*I* values (p*I* of 10.0 for CprB (Onaka et al., 1998)). The observation that KsbA, KsbB, and KsbC have p*I* values of 4.7, 5.4 and 5.6, respectively, is consistent with the results of phylogenetic analysis, implying that these three proteins are most likely to be a set of autoregulator receptors in *K. setae*. More detailed analysis of the amino acid sequences indicated that, although all of the KsbA/B/C proteins have the N-terminal helix-turn-helix DNA-binding domain and KsbA contains the residues Pro-117 and Trp-121, which are regarded as important for DNA binding and autoregulator binding, respectively (Onaka and Horinouchi, 1997; Sugiyama et al., 1998), KsbB has Ala at the position of Pro-117 with the conserved Trp-121, while KsbC contains Ser at the position of Trp-121 with the conserved Pro-117 (Fig. 2.2B). These findings suggested that KsbB and/or KsbC may be involved in the regulation of secondary metabolism, but most likely in a different manner from typical *Streptomyces* autoregulator receptors.

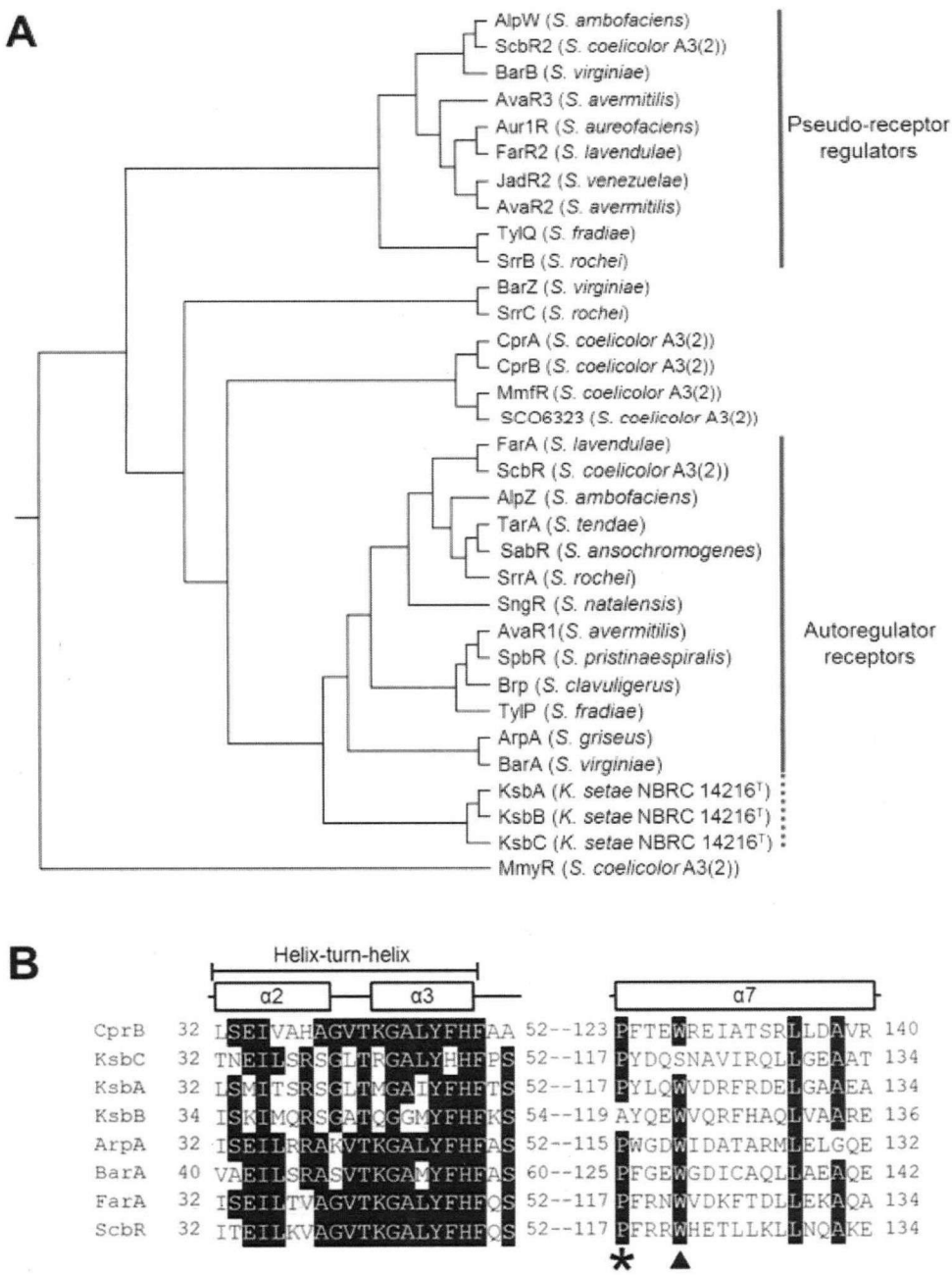

Fig. 2.2 Analysis of autoregulator receptors and receptor-like regulators with KsbC by phylogenetic tree (A) and amino acid alignment (B). (A) Phylogenetic tree constructed by the unweight pair group method with arithmetic mean. Multiple sequence alignment was conducted with the CLUSTALW program (http://www.genome.jp/tools/clustalw/). (B) Sequence alignment of the regions encoding helix-turn-helix DNA-binding domain and helices α7 of KsbC with those of autoregulator receptors. Black boxes indicate positions in the alignment at which the same amino acid is found in at least four of the seven sequences. The secondary structure elements of CprB are shown above its sequence. The numbers indicate the amino acid positions within each sequence. The asterisk and the filled triangle indicate an important residue for the binding to DNA and the formation of autoregulator-binding pockets, respectively.

The flanking regions of *ksbC* consist of two putative regulatory genes, *kse_44570* and *kse_44590* (Fig. 2.1B), with the former being homologous to various pseudo-receptor regulators, including ScbR2 of *S. coelicolor* A3(2) (37% identity), and the latter being homologous to various response-regulator proteins of a bacterial two-component signal transduction (TCS) system, including JadR1 of *Streptomyces venezuelae* (63% identity). The upstream region of the *kse_44590* has two putative autoregulator biosynthetic genes, *ksbS4* (*kse_44600*) and *ksbS5* (*kse_44610*), that encode proteins belonging to the AfsA-family proteins (including AfsA, which plays a role in A-factor biosynthesis (Kato et al., 2007)) and the NAD-dependent epimerase/dehydratase family (including BarS2, which plays a role in VB production (Lee et al., 2008)), respectively. The *ksbC* locus ranging from *kse_44570* to *ksbS5* closely resembles that of the VB-dependent regulatory island of *S. virginiae*, suggesting that, in addition to the previously reported KsbA-regulatory cascade, the *ksbC* locus is likely to form the second autoregulator-signaling cascade in *K. setae*.

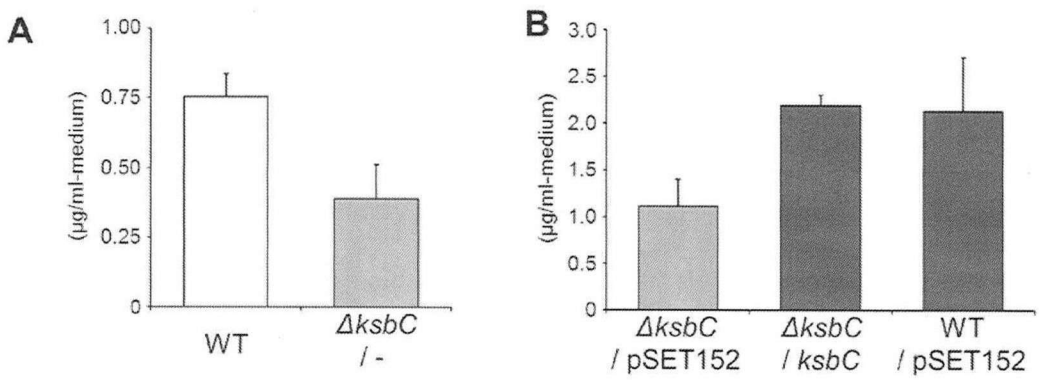

Fig. 2.3 Bafilomycin production in the ksbC disruptant. The amount of bafilomycin was the total amount of bafilomycin derivatives (bafilomycin A1, B1 and C1). Error bars represent standard deviations from triplicate experiments. WT, wild-type strain; Δ*ksbC*/-, *ksbC* disruptant; Δ*ksbC*/pSET152, Δ*ksbC* strain carrying pSET152; Δ*ksbC*/*ksbC*, *ksbC*-complemented Δ*ksbC* strain; WT/pSET152, the wild-type strain carrying pSET152. (A) Effect of *ksbC* disruption on bafilomycin production. (B) Complementation of the *ksbC* disruptant and effect of the pSET152-integration into the genome of *K. setae* on bafilomycin production.

2.3.2 KsbC acts as an activator of bafilomycin production and a repressor of kitasetaline production.

To assess the role of *ksbC* in the regulation of secondary metabolism, the *ksbC* gene was disrupted by insertion of a kanamycin-resistance gene, resulting in a *ksbC* disruptant. The

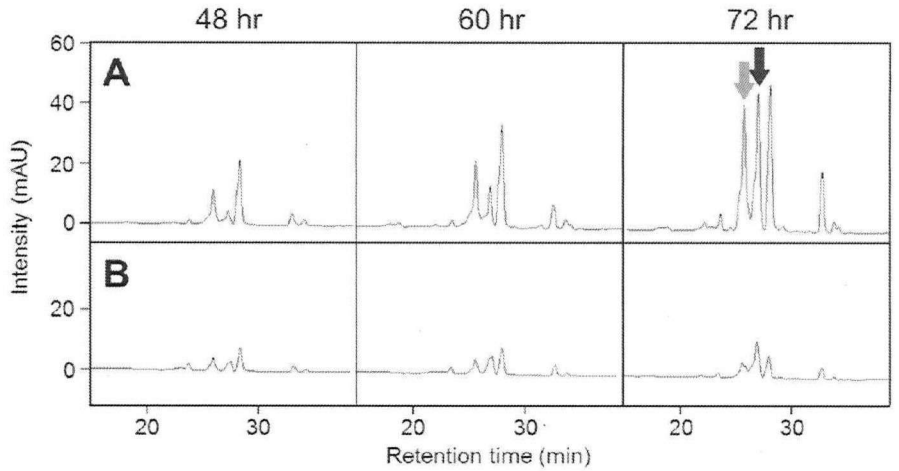

Fig. 2.4 Kitasetaline production in the *ksbC* disruptant (A) and the wild-type strain (B). HPLC chromatograms of methanol extracts from each strain cultivated at the indicated time. mAU, milli-absorbance units at 276 nm. The peaks of kitasetaline and JBIR-133 are indicated as a black arrow and a grey arrow.

bafilomycin production in the *ksbC* disruptant decreased to 52% of the wild-type levels at 5 days of cultivation (Fig. 2.3A). To confirm that the lowered bafilomycin production resulted solely from the *ksbC* disruption, the genome-integrated plasmid pLT436 containing an intact copy of *ksbC* and its upstream region was reintroduced into the *ksbC* disruptant (Fig. 2.3B). Although site-specific integration of an empty vector pSET152 (Bierman et al., 1992) alone (Δ*ksbC*/pSET152 vs Δ*ksbC*/-, or WT/pSET152 vs WT) increased the bafilomycin production (286% - 283% compared to that of the parental strain) for an unknown reason, it is clear that the *ksbC* disruption resulted in 52% reduction of the bafilomycin production and the *ksbC* complement recovered the production to the level of the corresponding wild-type strain.

These results confirmed that *ksbC* plays a positive role in the regulation of bafilomycin biosynthesis. Next, to investigate whether KsbC controls the production of other secondary metabolites, we examined the HPLC profiles of the methanol extract from the wild-type strain and the *ksbC* disruptant (Fig. 2.4). After 48 h of cultivation on solid medium, when the aerial mycelium started forming in the wild-type strain, several peaks (elution times: 26.5, 27.7, 28.7, and 33.5 min) from the *ksbC* disruptant were higher than those from the wild-type strain. This pattern continued throughout the entire cultivation period, and was observed until 15 days of cultivation. The differences became most apparent at 72 h of cultivation when the wild-type strain was in the early stage of sporulation but the *ksbC* disruptant aborted aerial mycelium formation (see below). After purification, 15 mg of pure compound eluted at 27.7 min (indicated by a black arrow in Fig. 2.4) was obtained from 1.25 liters of agar culture. Based on the physicochemical evidence, this compound [named kitasetaline (**2**) (Fig. 2.1A)] was a novel β-carboline alkaloid (Aroonsri et al., 2012a), which has not been isolated from any natural sources (see Chapter 3). The change of the kitasetaline production, attributable to the *ksbC* disruption, was partially restored by the introduction of the intact *ksbC* gene (Fig. 2.5). Thus, KsbC can be concluded to be a negative regulator for the initiation and the amount of kitasetaline production.

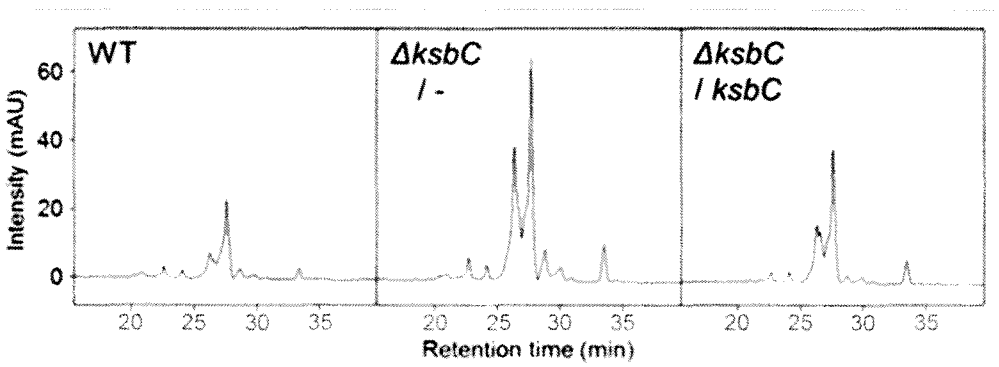

Fig. 2.5 Kitasetaline production in the ksbC-complemented ksbC mutant. HPLC chromatograms (detection at 276 nm) of methanol extracts from *K. setae* strains cultivated at 5 days are shown.

2.3.3 Regulation of bafilomycin and β-carboline biosynthesis

To elucidate the function of KsbC in transcriptional regulation of the gene clusters for bafilomycin and kitasetaline biosynthesis, the transcriptional levels were compared between the wild-type and the *ksbC* disruptant by semi-quantitative RT-PCR analysis or quantitative analysis as described in the Materials and methods. The biosynthetic gene cluster for bafilomycin is predicted to be composed of 18 genes from *kse_73410* to *kse_73580*, and the cluster includes *bfmR*, which is an essential pathway-specific positive regulator for the bafilomycin biosynthesis (H. Ikeda, unpublished data). Surprisingly, the transcriptional levels of *bfmR*, and biosynthetic genes for kitasetaline, *kse_70640*, and *kse_70630* (see Chapter 3), in the *ksbC* disruptant were nearly identical to those of the wild-type strain (data not shown). These results indicated that KsbC has a pleiotropic effect on the production of bafilomycin and kitasetaline without direct transcriptional control of these biosynthetic genes.

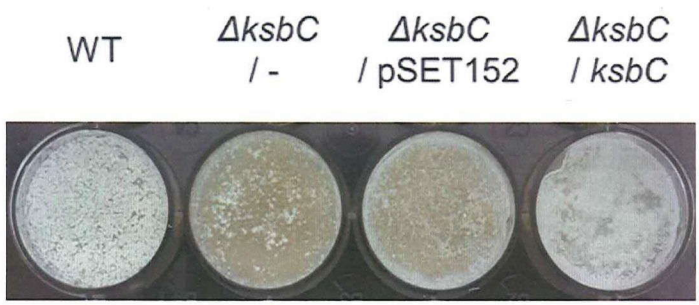

Fig. 2.6 Effect of *ksbC* disruption on the colony appearance. An equal volume of the mycelial seed culture was inoculated on ISP medium 4, and incubated for 5 days at 28°C.

2.3.4 Defect of aerial mycelium formation in the *ksbC* mutant

Although KsbA did not participate in the control of morphological development under the conditions employed herein (Choi et al., 2004), a few autoregulator receptors in *Streptomyces* spp. control not only antibiotic production but also morphological development

(Onaka et al., 1995; Folcher et al., 2001; Stratigopoulos et al., 2002). To investigate whether KsbC is involved in the morphological control of *K. setae*, we carefully examined the morphological characteristics of the *ksbC* disruptant on two different solid media. On ISP medium 2, the wild-type strain and the *ksbC* disruptant were found to have identical phenotypes (data not shown). On ISP medium 4, however, a defect in the aerial mycelium was observed for the *ksbC* mutant and the *ksbC* mutant carrying pSET152 at 5 days of cultivation when the wild-type strain and the Δ*ksbC/ksbC* strain showed aerial mycelium formation with an abundant amount of spores (Fig. 2.6). After 15 days of cultivation, the *ksbC* disruptants were still defective in the formation of the aerial mycelium (data not shown), indicating that KsbC is involved in morphological development, although the exact cascade or mechanism is at present unknown and will require detailed analysis in the future.

2.3.5 Binding of KsbC to the intergenic region of *kse_44590* and *ksbS4*

Many autoregulator receptors typically bind to ARE sequences that are found in the promoter region of the target genes (Folcher et al., 2001). A candidate 26-bases ARE-like sequence (5'-AAACATTACGGCGAAACTGTTTTTAC-3') is found in the 108-bases upstream region of the *ksbS4* gene (Fig. 2.7A), suggesting that KsbC may bind to the *ksbS4* upstream region. To examine whether KsbC has DNA-binding activity toward the *ksbS4* upstream region, we performed a gel-shift assay using a purified N-terminal His-tagged KsbC and DNA fragments encompassing the ARE-like sequence (Fig. 2.7B). Shift signals were only detected when this putative ARE sequence was included in the DNA probes, suggesting that KsbC probably recognizes the ARE-sequence upstream of *ksbS4*. As shown in Fig. 2.7C, this sequence, designated *ksbS4*-ARE, is highly similar to other ARE sequences that are present in the putative promoter region of the *afsA*-family genes, and two conserved sequences [5'-AAXAT(A/T)-3' and 5'-C(G/T)GTTTTT(T/A)-3'] emerged by the logo representation analysis. To investigate possible regulation of KsbC on the expression of

ksbS4 and/or *kse_44590*, we compared the transcriptional levels of these two genes between the wild-type strain and the *ksbC* disruptant. The mRNA level of *ksbS4* decreased significantly in the *ksbC* disruptant (45%) compared with that in the wild-type strain (Fig. 2.7D), while no remarkable differences in *kse_44590* transcription were observed (data not shown). These results, together with the results of the gel-shift assays, suggested that KsbC has a positive role on the transcriptional control of *ksbS4* through the binding to *ksbS4*-ARE in the *ksbS4* upstream region.

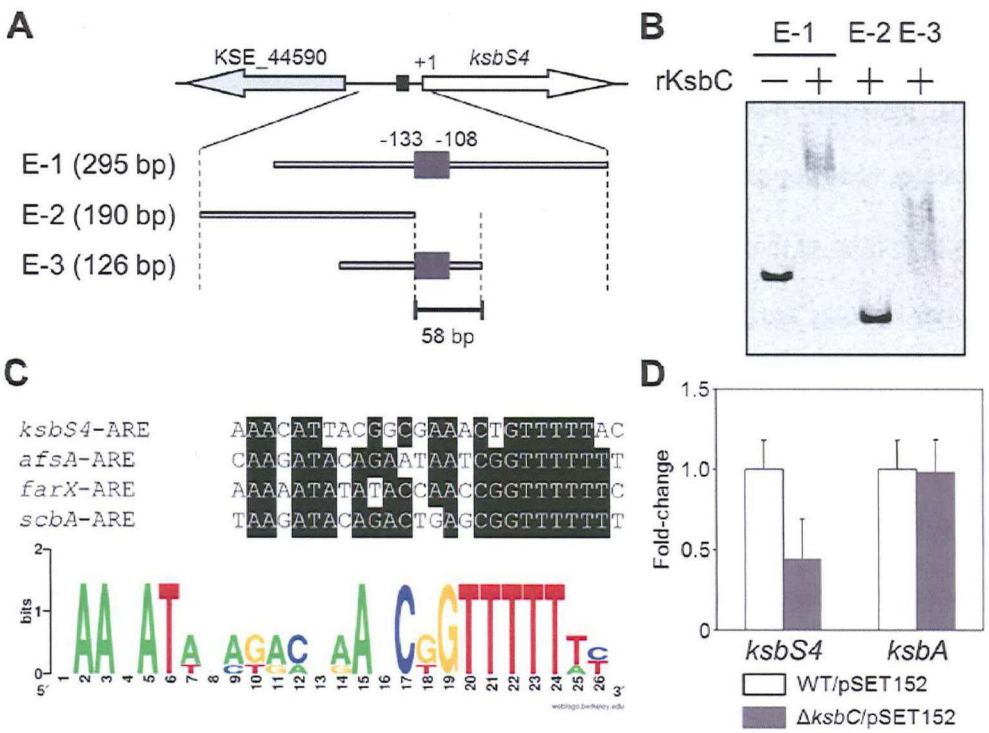

Fig. 2.7 Binding of KsbC to the intergenic region between *kse_44590* and *ksbS4* genes. (A) Location of probes used for the gel-shift assay. The probes E-1 to E-3 used in this work are shown and their lengths are indicated on the left. The location of the putative 26-bp ARE sequence, situated 108 bp upstream of the *ksbS4* gene, is indicated by a dark grey box. (B) Gelshift assay for the binding of purified His-tagged KsbC (rKsbC) to probes containing a plausible KsbC-binding site. (C) Comparison of the putative KsbC-binding sequence (*ksbS4*-ARE) with the ARE sequences located upstream of the *afsA*-family genes (upper panel) and a logo-representation for conserved nucleotides using computational methods (lower panel). Genes in the list are *afsA* of *S. griseus*; *farX* of *S. lavendulae* FRI-5; and *scbA* of *S. coelicolor* A3(2). The consensus nucleotides in the ARE sequences are highlighted by black boxes. The logo-representation was created using WebLogo analysis (http://weblogo.berkeley.edu/logo.cgi) based on the 26-bp ARE sequences. The relative sizes of the letters indicate their likelihood at the particular position. (D) Gene expression analysis of the *ksbS4* and *ksbA* genes by qRT-PCR. WT/pSET152, the wild-type strain carrying pSET152; Δ*ksbC*/pSET152, the Δ*ksbC* strain carrying pSET152. Fold-change is relative to the expression of each gene in the wild-type strain carrying pSET152. Error bars, SD from triplicate experiments.

2.4 Discussion

The γ-butyrolactone autoregulator receptors and their homologues play a pivotal role in the production of secondary metabolites in many *Streptomyces* species, and sometimes on morphological development. In most cases, a single autoregulator receptor is found in one *Streptomyces* strain, and it negatively regulates the production of a specific secondary metabolite by controlling the proximally situated biosynthetic gene cluster by adjusting the transcriptional level of the in-cluster *Streptomyces* antibiotic regulatory protein (SARP)-family gene (Stratigopoulos and Cundliffe, 2002; Aigle et al., 2005). Our laboratory previously provided the first report of an autoregulator receptor in non-*Streptomyces* actinomycetes that *K. setae* has an autoregulator-signaling cascade that uses KsbA as a negative regulator of bafilomycin production (Choi et al., 2004). In the present study, I verified that KsbC is the second autoregulator receptor of *K. setae* that has diverse regulatory functions on secondary metabolism (activation of bafilomycin and repression of kitasetaline and its derivative) in addition to a positive role on aerial mycelium formation which is not influenced by the first receptor KsbA. Two autoregulator receptors, KsbC and KsbA, have opposite properties for the regulation of bafilomycin production as the same target. The *ksbC* locus and the *ksbA* locus, neither of which have any biosynthetic gene cluster for secondary metabolites in the flanking regions, are 3.4 Mb and 1.8 Mb away from the biosynthetic gene cluster for bafilomycin, respectively, indicating that bafilomycin biosynthesis is controlled by two different autoregulator receptors encoded at a position distal to the gene cluster for bafilomycin biosynthesis. Transcriptional analysis demonstrated that KsbC does not control transcription of *bfmR*, which encodes a pathway-specific regulator for bafilomycin biosynthesis, and there appears to be no crosstalk between the KsbC- and KsbA-regulatory pathways for bafilomycin production (no difference of *ksbA* transcription in the *ksbC* disruptant (Fig. 2.7D) or *ksbC* transcription in the *ksbA* disruptant (data not shown)). These

findings indicated that, independent from the KsbA pathway, the KsbC pathway controls bafilomycin biosynthesis with no apparent control of BfmR.

With respect to the production of β-carboline alkaloids, we have identified plausible biosynthetic genes (*kse_70630* to *kse_70650*) for the formation of the β-carboline structure (see Chapter 3), which are 3.1 Mb and 1.5 Mb away from *ksbC* and *ksbA* loci, respectively. Unlike the case for the bafilomycin production, in the *ksbA* disruptant, the production level of kitasetaline and its derivatives including JBIR-133 was at the same level as in the wild-type strain (data not shown), indicating that only KsbC is involved in the regulation of the β-carboline alkaloids biosynthesis. Because the substrates/precursors for the β-carboline alkaloids biosynthesis are distinct from those for the bafilomycins biosynthesis, precursor competition is unlikely to be the underlying mechanism for the increase in β-carboline alkaloids production and the decrease in bafilomycin production in the *ksbC* disruptant, suggesting that KsbC is a specific negative regulator for β-carboline production without direct transcriptional control on the β-carboline biosynthetic genes. Thus, KsbC has bidirectional characteristics for secondary metabolism, as an activator in one pathway and a repressor in another pathway, together with regulation of morphological development. There have been few reports of autoregulator receptors exerting bidirectional characteristics like KsbC, except for FarA of the IM-2 signaling cascade in *Streptomyces lavendulae* FRI-5 (Kitani et al., 2001).

Disruption of the *ksbC* gene led to the discovery of new compounds such as kitasetaline and JBIR-133 together with the finding of a novel biosynthetic route for microbial β-carboline alkaloid, with the aid of a heterologous expression system in the genome-minimized *S. avermitilis* strain. Genomes of microorganisms, especially actinomycetes including *Streptomyces* species and *K. setae*, have numerous cryptic biosynthetic pathways in which genes appear to be expressed poorly, if at all, under the given cultivation condition.

From the viewpoint of identifying new compounds, the *K. setae* wild-type strain produces a negligible amount of kitasetaline and JBIR-133, while the *ksbC* disruptant shows precocious and abundant production of kitasetaline and JBIR-133, enabling identification of the structure. There have been only a few reports in which the deregulation of a biosynthetic pathway through the alteration of actinomycetes regulatory genes allows sufficient materials, especially novel natural compounds, to be purified for structure elucidation. In *S. coelicolor* A3(2), Gottelt and co-workers (2010) disrupted a pseudo-receptor regulator gene *scbR2* (Fig. 2.2A) located within a predicted biosynthetic gene cluster (*cpk*) and found a novel antibacterial activity of the unidentified product generated by the *cpk* gene cluster (Gottelt et al., 2010). In *S. ambofaciens*, Bunet and co-workers (2010) deregulated the pseudo-receptor regulator gene *alpW* (Fig. 2.2A) by gene disruption and identified that the *alp* gene cluster is responsible for the production of kinamycins (Bunet et al., 2010), although in both cases the targeted regulatory genes are located in a proximal silent gene cluster and the linkage between the new product and the biosynthetic gene cluster seems readily available. As in the case of KsbC, genetic manipulation of an autoregulator receptor (or pseudo-receptor regulator) that has no proximal gene cluster for secondary metabolites will be the next stage in the discovery of novel natural products and new biosynthetic pathways.

AfsA-family proteins are the key enzymes for biosynthesis of γ-butyrolactone autoregulators in *Streptomyces* species (Hsiao et al., 2007; Kato et al., 2007; Lee et al., 2010). The single *afsA*-family genes *afsA*, *scbA*, and *avaA* (Kitani et al., 2011) are present on the genomes of *S. griseus*, *S. coelicolor* A3(2), and *S. avermitilis*, respectively. The flanking region of *scbA* has *scbR* (a receptor of an SCB1 autoregulator), and that of *avaA* has *avaL1/L2* (receptors of an unidentified molecule), whereas the *afsA* locus is 3.9 Mb away from the locus of *arpA* (A-factor receptor gene). However, there have been many reports of *afsA*-family genes and receptor genes being clustered at the same locus in various

Streptomyces species (Nishida et al., 2007). Interestingly, the genome of *K. setae* has four copies of *afsA*-family genes, i.e., *ksbS4* in the *ksbC* locus, *ksbS2L/R* (*kse_01060t/kse_75680t*) in the two *ksbB* loci, and *ksbS3* (*kse_22970*) that is not accompanied by any autoregulator receptor gene. Together with the finding of two independent signaling pathways of KsbC and KsbA shown in the present study, these findings indicated that *K. setae* has multiple autoregulator signaling cascades for secondary metabolism, which is more complicated than the single cascade in *Streptomyces* species. As shown in Fig. 2.7C, the ARE sequences located in the upstream region of *afsA*-family genes are well-aligned, suggesting that regulatory mechanisms for autoregulator biosynthesis might be conserved in the autoregulator signaling cascade. The observation that the *ksbS4* transcription is down-regulated in the *ksbC* disruptant is similar to the finding in the *farA* mutant of *S. lavendulae* FRI-5, in which *farX*, an *afsA*-family gene for IM-2 biosynthesis, is not transcribed throughout the cultivation (Kitani et al., 2010). These phenomena indicate that fine-tuning systems to control autoregulator production are also conserved beyond the genus in actinomycetes.

2.5 Summary

In this chapter, I clarified the regulatory function of *ksbC* gene by gene disruption. The *ksbC* disruptant was deficient in aerial mycelium formation, as well as showed decrease in production of bafilomycin but increase in production of kitasetaline. As three phenotypes of the *ksbC* disruptant are changed, these suggested the pleiotropic regulation of KsbC in *K. setae*. Regarding to bafilomycin production, it is interesting that there are at least two regulations via KsbA and KsbC, which both regulators do not control the transcription of *bfmR* gene, a pathway specific regulator of bafilomycin biosynthetic gene cluster. Similarly, the transcriptions of biosynthetic genes of β-carboline are not affected by *ksbC* disruption,

demonstrating that KsbC does not control production of kitasetaline and its derivatives in the transcriptional level. The binding of rKsbC to ARE-like sequence located in the upstream region of *ksbS4* gene and result of qRT-PCR showing that transcription of *ksbS4* gene in the mutant was lower than in wild-type strain, suggested that KsbC might control the transcription of *ksbS4* gene.

Chapter 3

Kitasetaline, a novel β-carboline alkaloid from *Kitasatospora setae*, and identification of kitasetaline biosynthetic genes by using heterologous expression

3.1 Introduction

Kitasatospora setae NBRC 14216^T produces bafilomycins A1 and B1 (**1**), specific inhibitors of vacuolar H^+-ATPase (Bowman et al., 1988; Otoguro et al., 1988). The complete genome sequence of *K. setae* revealed that this microorganism seems to have at least 24 genes or gene clusters for the biosynthesis of secondary metabolites and the vast majority of these genes and clusters are of unknown function in the biosynthetic process and are presumably cryptic biosynthetic pathways (Ichikawa et al., 2010). These findings suggest that the genus *Kitasatospora* is a promising reservoir of bioactive natural products. With these cryptic pathways in which genes appear to be expressed poorly or not at all under the normal genetic and cultivation conditions, the activation of any of them could lead us to the discovery of novel natural products.

γ-Butyrolactone-autoregulator signalling cascades in actinomycetes, especially in streptomycetes, are known to be the major regulatory system for the production of secondary metabolites (Bibb, 2005; Takano, 2006). *K. setae* harbors three autoregulator receptor homologues: KsbA, a negative regulator of bafilomycin production (Choi et al., 2004); KsbB (KSE01050t and KSE_75690t, which are identical genes in the terminal inverted repeats at both ends of the linear chromosome) (Ichikawa et al., 2010); and KsbC (KSE_44580) (Ichikawa et al., 2010), whose function remains to be clarified. In the course of investigating metabolites to understand regulatory systems for secondary metabolism in *K. setae*, one or

more remarkably large peaks were found from the agar culture of *K. setae* Δ*ksbC* mutant (Aroonsri et al., 2012b) (Fig. 2.4), whose *ksbC* gene was disrupted by insertion of a kanamycin-resistance gene cassette, compared with that in the wild-type strain. Therefore, we isolated and elucidated the structure of the major peak eluded at 27.7 min (indicated by black arrow in Fig. 2.4) from the agar culture of *K. setae* Δ*ksbC* mutant. Moreover, we also identified the biosynthetic genes of kitasetaline and its derivatives JBIR-133 and JBIR-134 as new compounds by heterologous expression in a *Streptomyces* host strain, and suggested a possible route for the supply of the β-carboline structure in bacteria.

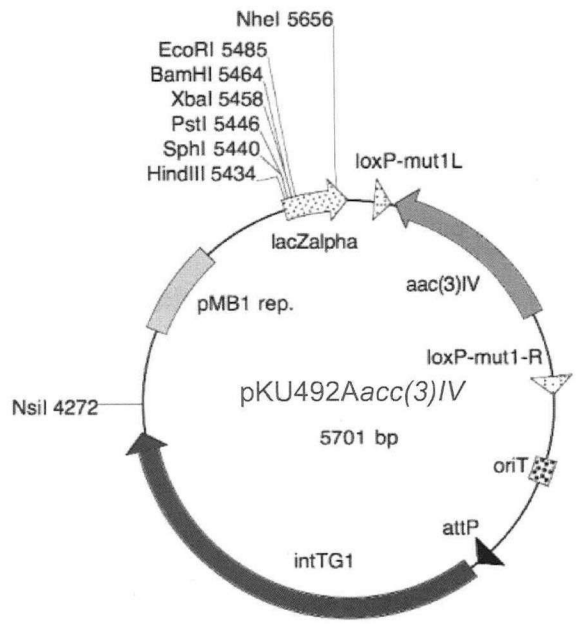

Fig. 3.1 A restriction map of pKU492A*aac(3)IV* used in this study.

3.2 Materials and methods

3.2.1 Bacterial strains, plasmids, and growth conditions

K. setae NBRC 14216^T from the NITE Biological Resource Center (NBRC), Japan, was

grown on ISP medium 2 (Becton Dickinson and Company, Franklin Lakes, NJ, USA). *E. coli* GM2929 *hsdS*::Tn*10* was used to prepare unmethylated DNA for protoplast transformation in *Streptomyces avermitilis* SUKA22 (isogenic to *S. avermitilis* SUKA17 (Komatsu et al., 2010) but *loxP* sequences were replaced to mutant-*loxP* sequences). The genome-integrated vectors, pKU492A*aac(3)IV* (Fig. 3.1) and pKU503 (Komatsu et al., 2010), were used for subcloning a gene cluster for kitasetaline biosynthesis and the construction of BAC library for *K. setae* genome. Spores (1 x 10^8 CFU) *K. setae* Δ*ksbC* mutant were inoculated into 500-ml baffled flasks containing 70 ml of YMM medium consisting of yeast extract 0.4%, malt extract 1%, and maltose 0.4% (pH 7.4). The flasks were cultivated on a reciprocal shaker (120 spm) at 28°C for 36 hr. The seed culture was inoculated on YMD4 medium consisting of yeast extract 0.4%, malt extract 1%, dextrin 4%, and agar 2% (pH 7.4), and grown for 5 days at 28°C.

3.2.2 Purification and structure elucidation of kitasetaline

The 1.25 L agar culture was diced and extracted with 2 volumes of MeOH. After the MeOH extract was filtered and concentrated, the dark-brownish oily residue was subjected to reversed-phase column chromatography using a Sep-Pak Vac 35 cm^3 (10 g) C_{18} cartridge (Waters, Milford, MA) with a MeOH-H_2O step gradient system (0:1, 1:9, 2:8, and 4:6 v/v). The 20% MeOH fraction was subjected to repeated reversed-phase HPLC on a CAPCELL-PAK C_{18} column (UG80; 5 μm; 10 i.d. x 250 mm; Shiseido, Tokyo, Japan) with a linear gradient system [eluents: H_2O containing 0.075% trifluoroacetic acid (TFA) (A), MeOH containing 0.01% TFA (B); gradient 0-3 min 34% B, 3-21 min 34% B to 40% B, 21-24 min 40% B, and 24-25 min 40% B to 34% B; flow rate 3.5 ml/min; UV detection at 276 nm], to yield 15 mg of purified compound (**2**) (Fig. 2.1A). The HRFABMS spectrometry (positive mode) was recorded on a JEOL JMS-700 spectrometer. UV and IR spectra were recorded on a Hitachi U-3210 spectrophotometer and an FTIR-8400S (Shimadzu, Kyoto, Japan), respectively. The optical rotation data were recorded on a P-1020 polarimeter (JASCO,

Tokyo, Japan). The NMR spectra were obtained on a Varian Inova 600 MHz NMR system. Physicochemical properties of kitasetaline are as follows; kitasetaline (**2**): yellow oil; UV (methanol) λmax (log ε) 218 (4.35), 238 (4.42), 271 (4.65), 305 (4.05), and 379 (3.50) nm. IR (KBr) ν_{max} of 3066, 1610, 1369, 1247, and 752 cm^{-1}; HRFABMS *m/z* 402.1125 $[M+H]^+$ (calcd. for $C_{19}H_{20}N_3O_5S$, 402.1124); ^{13}C NMR (150 MHz, DMSO-d_6, δ ppm): 143.5 (C-1), 136.6 (C-3), 115.9 (C-4), 122.3 (C-5), 120.4 (C-6), 128.7 (C-7), 112.4 (C-8), 136.0 (C-10), 127.9 (C-11), 121.4 (C-12), 141.0 (C-13), 33.7 (C-14), 30.2 (C-15), 166.8 (C-16), 172.4 (C-1'), 52.4 (C-2'), 33.2 (C-3'), 169.6 (C-5'), 22.4 (C-6'). ^1H NMR (600 MHz, DMSO-d_6, δ ppm): 8.78 (1H, *s*, H-4), 8.35 (1H, *d*, *J*=7.9 Hz, H-5), 7.30 (1H, *t*, *J*=7.6 Hz, H-6), 7.59 (1H, *t*, *J*=7.6 Hz, H-7), 7.64 (1H, *d*, *J*=8.2 Hz, H-8), 3.40 (2H, *t*, *J*=7.6 Hz, H-14), 3.12 (2H, *td*, *J*=7.6, 1.8 Hz, H-15), 4.42 (1H, *ddd*, *J*=8.5, 8.2, 5.0 Hz, H-2'), 2.86 (1H, *dd*, *J*=13.5, 8.5 Hz, H-3'), 3.00 (1H, *dd*, *J*=13.7, 5.0 Hz, H-3'), 8.26 (1H, *d*, *J*=8.2 Hz, H-4'), 1.84 (3H, *s*, H-6').

3.2.3 Molecular cloning of the gene cluster for kitasetaline biosynthesis and evaluation of the gene cluster by heterologous expression

The mycelia of *K. setae* were embedded into 0.6 % SeaPlaque GTG agarose (Lonza Group Ltd. Switzweland) before digestion with 1 mg/ml of lysozyme for 16 h at 37°C. The resulting protoplasts were lysed by addition of 1% sodium *N*-lauroylsarcosinate and 1 mg/ml of proteinase K at 50°C for 24 h. After inactivation of proteinase K by 0.1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (Nacalai tesque Inc. Japan) and removal of sodium *N*-lauroylsarcosinate by repeated washing with 50 mM EDTA (pH 8.0), DNA embedded in the agarose plug was partially digested with *Bam*HI. From 100 to 150-kb fragments were purified by CHEF electrophoresis. The agarose gel containing DNA fragments were molten by NaI with half weight of gel and molten agarose and NaI were removed by dialysis against TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The residual agarose were completely molten by GELase Enzyme preparation (Illumina Inc. USA). The

DNA fragments were ligated with *Bam*HI fragment of pKU503 and the ligated DNA was transformed into *E. coli* NEB10β. Each BAC clone was stored in six 384-well plates containing LB (100 μg/ml ampicillin and 20 % glycerol) at -80°C. A BAC clone, pKU503facP2-L12, containing putative gene cluster for kitasetaline biosynthesis, was introduced into *S. avermitilis* SUKA22 by protoplast transformation. For minimization of a gene cluster for kitasetaline biosynthesis, about 500-bp homologous regions at upstream and downstream of the gene cluster were prepared by PCR with pKU503facP2-L12 as a template using two primer pairs corresponding to upstream and downstream of the gene cluster, upstream primer pair, forward: 5'-CTCGAG**TCTAGA***AGTTCTTCGACCTCGGCCTCACCTCC*-3' (Bold characters indicate *Xba*I site, and italic characters correspond to the region from 8,040,461 to 8,040,486 nt of *K. setae*.) and reverse: 5'-CTCGAG**GGATCC***GGACGCTGGAGCAGATGAAGGACT*-3' (Bold characters are *Bam*HI site and italic characters correspond to the region from 8,040,873 to 8,040,897 nt of *K. setae*.), and downstream primer pair, forward: 5'-CTCGAG**GGATCC***CGGGCTGCGCCGCATGGACCTC*-3' (Bold characters indicate *Bam*HI site and italic characters correspond to the region from 8,051,125 to 8,051,148 nt of *K. setae*.) and reverse: 5'-CTCGAG**AAGCTT***CAGGGACGGCGCCTGCCACAGCTCGAAGG*-3' (Bold characters are *Hin*dIII site and italic characters correspond to the region from 8,051,601 to 8,051,573 nt of *K. setae*.), respectively. The minimum cloning vector pRED (21) lacking *Xba*I site was also amplified by PCR using the primer pairs, forward: 5'-CTCGAG**TCTAGA**TGCCAGGAAGATACTTAACAG-3' (Bold characters indicate *Xba*I site.) and reverse: 5'-CTCGAG**AAGCTT**CCATTCATCCGCTTATTATC-3' (Bold characters are *Hin*dIII site.). After amplification of above segments, all segments treated with *Dpn*I to remove a template DNA, the upstream segment digested with *Xba*I/*Bam*HI, the downstream segment digested with *Bam*HI/*Hin*dIII, and the vector segment digested with

*Xba*I/*Hin*dIII were ligated together to yield pRED::upstream::downstream. The resulting plasmid was amplified by PCR with the forward primer of the downstream segment and the reverse primer of the upstream segment. The amplified fragment was treated with *Dpn*I to remove a template DNA and digested with *Bal*31 for 30 sec at 30°C. The biosynthetic gene cluster for kitasetaline in the pKU503facP2-L12 was replaced by a linear molecule of pRED::upstream::downstream using the homologous regions using the λRED recombination system (*E. coli* DH10B and pKD119 were used (Komatsu et al., 2010). to generate pRED::*ksl* (kitasetaline) cluster (11,141-bp; corresponds from 8,040,461 to 8,051,601 nt of *K. setae*). A *Xba*I/*Hin*dIII fragment including 11.1-kb *ksl* cluster, recovered from pRED::*ksl*, was inserted into the *Xba*I/*Hin*dIII sites of pKU492A*aac(3)IV*, resulting in pKU492A*aac(3)IV*::*ksl* cluster, named pLT437. pLT437 was digested with either *Hin*dIII/*Bsu*36I, *Hin*dIII/*Eco*RV or *Xba*I/*Bst*BI, treated with T4 DNA polymerase to yield blunt ends, and self-ligated, resulting in pLT438, pLT439, or pLT440, respectively. The constructed plasmids were transferred into *E. coli* GM2929 *hsdS*::Tn*10* and the unmethylated plasmids were introduced into *S. avermitilis* SUKA22 by protoplast transformation. Integration of the plasmids was confirmed by apramycin resistance and PCR analysis. Spores (1 x 10^8 CFU) of SUKA22 derivative strains were inoculated on 2.5 ml YMS-MC medium, and incubated at 28°C for 4 days. The analysis of the products was described in "Purification and structure elucidation of kitasetaline".

3.2.4 Isolation and structural elucidation of novel kitasetaline analogues

The SUKA22 carrying pKU503facP2-L12 was cultivated in a 15-ml test tube containing 5 ml of seed medium (Cane et al., 2006). The test tube was incubated on a reciprocal shaker at 27°C for 2 days (320 rpm). An aliquot (2.5 ml) of the culture was then transferred to 500-ml baffled Erlenmeyer flasks each containing 100 ml of a production medium (Cane et al., 2006). The sample was cultured on a rotary shaker (180 rpm) at 27°C for 5 days. After the

fermentation broth (100 ml) was separated by centrifugation, the mycelial cake was extracted with acetone (40 ml) and concentrated *in vacuo*. The aqueous concentrate was successively washed with ethyl acetate and extracted with *n*-butanol (20 ml × 3), after which the *n*-butanol layer was evaporated to dryness. The supernatant was washed with ethyl acetate and then extracted with *n*-butanol (50 ml × 3), after which the organic phase was evaporated. The combined extracts (40.2 mg) were subjected to reversed-phase MPLC eluted with a stepwise solvent system of water/methanol (100:0, 90:10, 80:20, 60:40, 40:60, 20:80, 0:100). The fraction eluted with 10% methanol (12.3 mg) was further purified by RP-HPLC on a CAPCELL PAK C18 MGII column (5.0 μm, 20 i.d. × 150 mm) developed with 35% aqueous methanol containing 0.1% formic acid (flow rate: 10 ml/min) to yield **3** (1.2 mg, retention time = 27.4 min). Next, the 20% methanol eluate (2.0 mg) was purified by RP-HPLC on a CAPCELL PAK C18 MGII column (5.0 μm, 10 i.d. × 150 mm) developed with a gradient solvent system of aqueous methanol (40-55% for 30 min) containing 0.1% formic acid (flow rate: 4 ml/min) to yield **4** (0.5 mg, retention time = 26.3 min). UV and IR spectra were measured on a Beckman Coulter DU730 UV/vis spectrophotometer and a Horiba FT-720 spectrophotometer, respectively. NMR spectra were recorded on a Varian NMR System 600 NB CL. Chemical shifts were calibrated internally against the residual signal of the solvent in which the sample was dissolved (DMSO-d_6: δ_C 39.7, δ_H 2.49). HRESIMS data were recorded using a Waters LCT-Premier XE mass spectrometer. MPLC was carried out on a Purif-Pack ODS-100 column (Shoko Scientific). RP-HPLC was carried out using a CAPCELL PAK C18 MGII column (5.0 μm, 20 i.d. × 150 mm, 10 i.d. × 150 mm; Shiseido) with a Waters 2996 photodiode array detector and a Waters 3100 mass detector. Physico-chemical properties were as follows; **JBIR-133 (3)**: pale yellow amorphous solid; UV (methanol) λmax (log ε) 238 (4.41), 267 (4.57), 302 (3.97), 376 (3.41) nm; IR (KBr) ν_{max} 3143, 1708, 1241 cm^{-1}; HRESIMS *m/z* 285.0869 $[M+H]^+$ (calcd. for $C_{15}H_{13}N_2O_4$, 285.0875); ^{13}C NMR (150 MHz,

DMSO-d_6, δ ppm): 144.0 (C-1), 135.3 (C-3), 115.7 (C-4), 122.3 (C-5), 120.3 (C-6), 128.6 (C-7), 112.5 (C-8), 135.9 (C-10), 128.6 (C-11), 121.5 (C-12), 141.4 (C-13), 28.3 (C-14), 31.8 (C-15), 166.8 (C-16), 174.2 (15-CO_2H). ^1H NMR (600 MHz, DMSO-d_6, δ ppm): 8.76 (1H, *s*, H-4), 8.34 (1H, *d*, *J*=7.8 Hz, H-5), 7.29 (1H, *t*, *J*=7.8 Hz, H-6), 7.58 (1H, *t*, *J*=7.8 Hz, H-7), 7.64 (1H, *d*, *J*=7.8 Hz, H-8), 12.12 (1H, *s*, H-9), 3.39 (2H, *t*, *J*=7.2 Hz, H-14), 2.91 (2H, *t*, *J*=7.2 Hz, H-15). **JBIR-134 (4)**: pale yellow amorphous solid; UV (MeOH) λmax (log ε) 237 (4.44), 269 (4.60), 302 (4.07), 377 (3.62) nm; IR (KBr) ν_{max} 3062, 1701, 1234 cm^{-1}; HRESIMS *m/z* 241.0974 $[M+H]^+$ (calcd. for $C_{14}H_{13}N_2O_2$, 241.0977); ^{13}C NMR (150 MHz, DMSO-d_6, δ ppm): 146.7 (C-1), 135.3 (C-3), 115.7 (C-4), 122.3 (C-5), 120.3 (C-6), 128.6 (C-7), 111.2 (C-8), 135.6 (C-10), 127.6 (C-11), 121.6 (C-12), 141.4 (C-13), 26.9 (C-14), 12.9 (C-15), 167.1 (C-16). ^1H NMR (600 MHz, DMSO-d_6, δ ppm): 8.76 (1H, *s*, H-4), 8.33 (1H, *d*, *J*=7.8 Hz, H-5), 7.28 (1H, *t*, *J*=7.8 Hz, H-6), 7.57 (1H, *t*, *J*=7.8 Hz, H-7), 7.64 (1H, *d*, *J*=7.8 Hz, H-8), 12.02 (1H, *s*, H-9), 3.17 (2H, *q*, *J*=7.4 Hz, H-14), 1.38 (3H, *t*, *J*=7.4 Hz, H-15).

3.3 Results

3.3.1 Kitasetaline, a novel β-carboline alkaloid from *Kitasatospora setae*

In Chapter 2 (section 2.3.2), in order to examine the production of secondary metabolites other than bafilomycin, the methanol extract from the wild-type strain and the *ksbC* disruptant were subjected to gradient HPLC. As shown in Fig 2.4, several peaks (elution times: 26.5, 27.7, 28.7, and 33.5 min) from the *ksbC* disruptant were larger than those from the wild-type strain. After purification, 15 mg of pure compound eluted at 27.7 min (indicated by a black arrow in Fig. 2.4) was obtained from 1.25 l of agar culture and afterward analyzed by spectroscopic analysis as follows. The HRFABMS spectrometry showed a molecular ion peak at *m/z* 402.1125 $[M+H]^+$, corresponding to a molecular formula

of $C_{19}H_{19}N_3O_5S$, which indicated the presence of 12 unsaturation sites in the structure. The UV spectrum of kitasetaline (**2**) showed λ_{max} as 218, 238, 271, 305, and 379, which agreed well with the characteristic absorptions of a β-carboline chromophore at 238, 271, 305, and 379 nm (Ohmoto et al., 1981). The IR absorption bands suggested the presence of a secondary amide group (3066 and 1610 cm^{-1}) and an acetyl group (1369 cm^{-1}). The NMR spectra showed signals assignable to two methylene protons [δ_H 3.40, 3.12 (H_2-14 and H_2-15)] and five aromatic protons. The ^1H-^1H correlations demonstrated three partial structures: C-5–C-6–C-7–C-8, suggesting the unsubstituted aromatic ring A of β-carboline basic skeleton; C-14–C-15; and C-3′–C-2′–N-4′. In the HMBC experiment, long-range HMBC correlations were observed between the following proton and carbon pairs: H-4 (δ_H 8.78) and C-16 (δ_C 166.8), indicating that the COOH was attached at C-3 (δ_C 136.6); and H_2-14 and C-10 (δ_C 136.0) as well as H_2-15 and C-1 (δ_C 143.5), indicating that a C-14–C-15 fragment of kitasetaline was connected at C-1. These correlations indicated the presence of a 1-ethyl β-carboline 3-carboxylic acidic moiety. The HMBC cross peaks from H-2′ (δ_H 4.42) to C-1′ (δ_C 172.4) and C-5′ (δ_C 169.6) and from H_3-6′ (δ_C 22.4) to C-5′, along with the ^1H-^1H correlation of C-3′–C-2′–N-4′, demonstrated the presence of *N*-acetylcysteine. The aliphatic methine proton (H_2-3′) showed an HMBC correlation to the methylene carbon C-15 in the 1-ethyl β-carboline 3-carboxylic acidic moiety. In addition, the cross peak from H_2-15 to C-3′ was also observed. Taken together with chemical shifts of C-3′, these results suggested a connection between the 1-ethyl β-carboline 3-carboxylic acidic moiety and *N*-acetylcysteine via a thioether linkage. Thus, we elucidated the planar structure of **2** as 1-(2-((2-acetamido-2-carboxyethyl)thio)ethyl)-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid, shown in Fig. 3.2, which has not been isolated from any natural sources.

To estimate the absolute configuration of **2**, the optical rotation data were recorded, demonstrating that the $[\alpha]_D$ value was negative ($[\alpha]^{25}_D$ -13.7 (*c* 0.13, MeOH)). $[\alpha]_D$ values are

negative for typical L-cysteine derivatives, which have the *R* configuration: *N*-acetyl-*S*-methyl-L-cysteine, $[\alpha]^{21}_{D}$ -30.1 (*c* 1.0, H_2O) (Meese et al., 1990); *N*-acetyl-*S*-ethylcysteine, $[\alpha]^{21}_{D}$ -27.0 (Thomson et al., 1963); (2*R*)-2- acetamido-3-(methylthiocarbamoylsulfanyl)propanoic acid, $[\alpha]^{21}_{D}$ -35.0 (Vermeulen et al., 2003). These

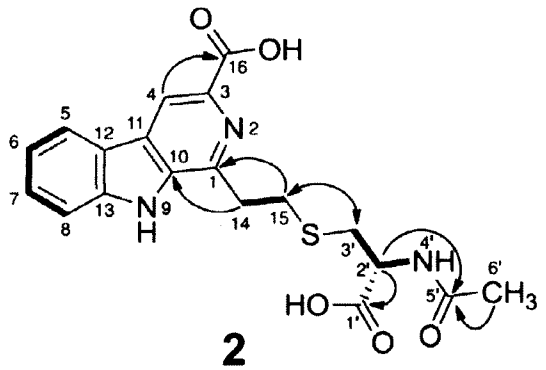

Fig. 3.2 Chemical structure of kitasetaline (**2**). COSY and HMBC correlations of kitasetaline (**2**) are indicated by bold lines and arrows, respectively.

findings suggested that compound **2** should have the absolute configuration of 2'*R*. Thus, the plausible structure of **2** was elucidated as 2'*R*-*N*-acetylcysteinyl-*S*-ethyl-β-carboline-3-carboxylic acid [(*R*)-1-(2-((2-acetamido-2-carboxyethyl)thio)ethyl)-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid], and was named kitasetaline (for *Kitasatospora setae* β-carboline alkaloid).

Finally, kitasetaline was assayed for biological activity against different targets, such as antimicrobial activity (methicillin-resistant *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Candida albicans*) at the 10 μg/disk level, nematocidal activity, and effect on the cell-cycle status of Jurkat cells up to at least 25 μM (Koizumi et al., 2004). However, kitasetaline (**2**) showed no activity against the investigated targets.

3.3.2 Heterologous expression and identification of the kitasetaline biosynthetic genes

We recently initiated a screening program with a genomic BAC library of *K. setae* to discover cryptic natural compounds. Each BAC clone carrying a gene cluster for secondary metabolite biosynthesis estimated by bioinformatics analysis was introduced by protoplast transformation into a large-deletion mutant of *S. avermitilis*, SUKA22, a model host for heterologous expression of secondary metabolism, and their production profiles were explored by HPLC/DAD analysis. In the course of our extensive search for secondary

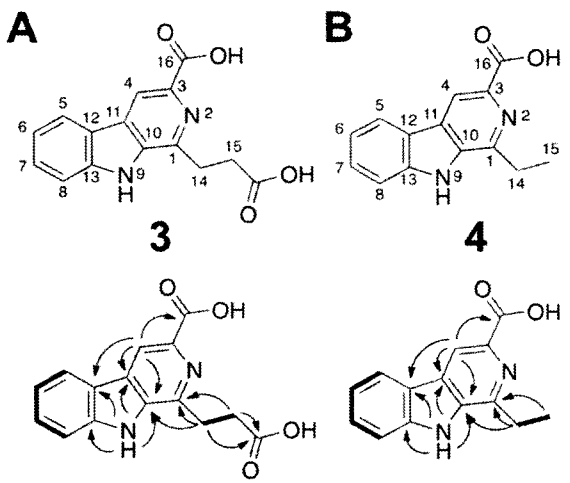

Fig. 3.3 Chemical structures of JBIR-133 (A; **3**) and JBIR-134 (B; **4**). Lower column shows COSY and HMBC analysis. Bold lines represent 1H-1H correlations and arrows show key HMBC correlations (1H <-> 13C).

metabolites, *S. avermitilis* SUKA22 carrying pKU503facP2-L12 exhibited a couple of peaks on ODS-HPLC analysis, which showed the characteristic absorptions of a β-carboline chromophore. As expected, no corresponding peak was observed in SUKA22 carrying pKU503 (empty vector) as a negative control. From 100 ml culture of the SUKA22 carrying pKU503facP2-L12, two major components, kitasetaline (**2**) and JBIR-133 (**3**), and a minor

component, JBIR-134 (**4**) were detected and purified. The UV spectra of **3** and **4** well agreed with the characteristic absorptions of a β-carboline chromophore at 238, 271, 305 and 379 nm, which were the same as those of kitasetaline (**2**). The IR absorptions (1708 and 1701 cm^{-1}, respectively) suggested the presence of carboxyl groups. The structure of **3** was mainly established by the analyses of the series of NMR spectra such as DQF-COSY, HSQC and CT–HMBC. Eleven aromatic carbons (C1-C13) were assignable to the β-carboline chromophore. The remaining substructure was elucidated as follows. The ^{1}H-^{1}H coupling between two methylene protons H_2-14 (δ_H 3.39) and H_2-15 (δ_H 2.91), which in turn ^{1}H-^{13}C long-range coupled to a nitrogen-bonded aromatic quaternary carbon C-1 (δ_C 144.0) and a carbonyl carbon 15-COOH (δ_C 174.2) established that a propionic acid moiety substituted at C-1. The ^{1}H-^{13}C long-range correlation from an aromatic proton H-4 (δ_H 8.76) to a carbonyl carbon C-16 (δ_C 166.8) together with the molecular formula indicated that the COOH group was attached to an aromatic quaternary carbon C-3 (δ_C 135.3). These results identified the structure of **3** as 1-(2-carboxyethyl)-β-carboline-3-carboxylic acid (Fig. 3.3A). The 1D and 2D NMR data of a minor component (**4**) led us to determine the substructure, β-carboline-3-carboxylic acid moiety, as same as that of **3**. The remaining partial structure was determined as follows. The ^{1}H-^{1}H coupling between methylene protons H_2-14 (δ_H 3.17) and methyl protons H_3-15 (δ_H 1.38), which in turn ^{1}H–^{13}C long-range coupled to a nitrogen-bonded aromatic quaternary carbon C-1 (δ_C 146.7), established an ethyl moiety substituted at C-1. Thus, the structure of **4** was determined as 1-ethyl-β-carboline-3-carboxylic acid (Fig. 3.3B). These two β-carboline alkaloids **3** and **4** have not been isolated from any natural sources. Comparison of the UV/visible spectra and the retention time of JBIR-133 (**3**) to those of the production profile of the *K. setae ksbC* mutant revealed that the *ksbC* disruption resulted in the production of JBIR-133 (elution time at 26.5 min, indicated by a grey arrow in Fig. 2.4).

To identify genes responsible for the biosynthesis of kitasetaline and JBIR-133, a series

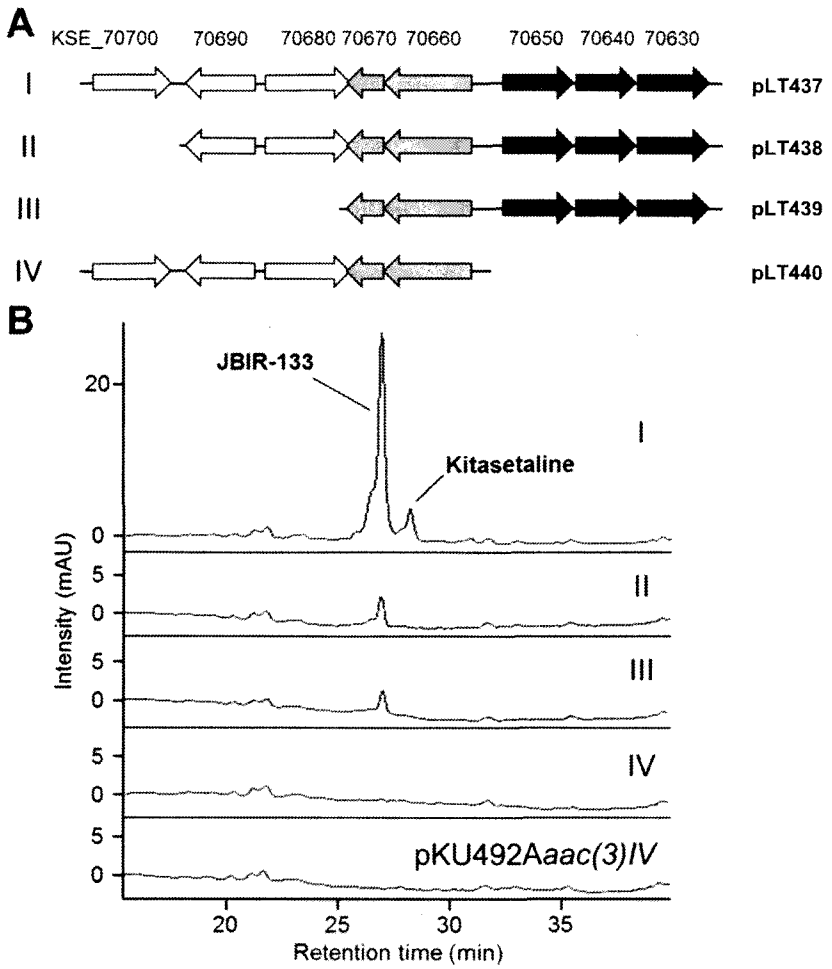

Fig. 3.4 Heterologous expression of the β-carboline gene cluster in *S. avermitilis* SUKA22 and determination of the minimal gene cluster. (A) Schematic representation of the fragments used in this experiment. I: pLT437 containing genes from *kse_70630* to *kse_70700*; II: pLT438 containing genes from *kse_70630* to *kse_70690*; III: pLT439 containing genes from *kse_70630* to *kse_70670*; IV: pLT440 containing genes from *kse_70660* to *kse_70700*. The genes indicated by arrows are completely included in each fragment. (B) HPLC analyses (276 nm) of the metabolite profiles of *S. avermitilis* SUKA22 carrying pLT437 (I), pLT438 (II), pLT439 (III), pLT440 (IV) and pKU492A*aac(3)IV* as an empty vector, respectively. JBIR-133 and kitasetaline are eluted at 26.7 min and 28.0 min, respectively.

of pKU492A*aac(3)IV* containing shortened fragments from pKU503facP2-L12 was constructed (Fig. 3.4A), yielding pLT437-pLT440, which was then introduced into *S. avermitilis* SUKA22. After fermentation on solid medium, methanol-extracts were subjected to HPLC analysis (Fig. 3.4B). SUKA22 carrying pLT437 (harboring eight genes, *kse_70630* to *kse_70700*) produced kitasetaline as a minor metabolite and JBIR-133 as a major

metabolite (Fig. 3.4B, trace I). However, deletion of *kse_70700* (as in pLT438) led to a remarkable decrease of JBIR-133 production and no production of kitasetaline (Fig. 3.4B, trace II). Although SUKA22 carrying pLT439 still showed JBIR-133 production at a yield comparable to that of SUKA22 carrying pLT438 (Fig. 3.4B, trace III), deletion of three genes (*kse_70630* to *kse_70650*), probably forming a single transcriptional unit (see below), failed to produce JBIR-133, which is similar to the production profile of SUKA22 carrying pKU492A*aac(3)IV* as a negative control (Fig. 3.4B, traces IV and pKU492A*aac(3)IV*). Based on these experimental evidences, it estimates that *kse_70630*, *kse_70640*, and *kse_70650* would be mainly concerned with the formation of the β-carboline moiety in the biosynthesis of kitasetaline and its derivative, JBIR-133.

3.3.3 Organization of genes involved in β-carboline biosynthesis

Three genes (*kse_70630*, *kse_70640*, and *kse_70650* for the biosynthesis of β-carboline structure) are divergently located from the downstream *kse_70620* encoding putative type I polyketide synthase and the upstream *pabB* (*kse_70660*) encoding putative *p*-aminobenzoate synthase. Interestingly, with narrow intergenic regions (48 bp between *kse_70630* and *kse_70640*, and 74 bp between *kse_70640* and *kse_70650*), the three genes seem to be transcribed as a polycistron. The predicted 392-aa gene product of *kse_70630* shows 37% identity and 57% positive matches to CypX of *Ktedonobacter racemifer* DSM 44963 (ZP_06966380), 36% identity and 55% positive matches to CypX of *Paenibacillus* sp. Y412MC10 (YP_00324629) and 39% identity and 55% positive matches to Orf1, a probable cytochrome P450 monooxygenase, of *Streptomyces eurythermus* (ABV49609). The predicted 317-aa KSE_70640 shows moderate similarity to gene products of Gram-negative bacteria, *Burkholderia* and *Agrobacterium* species; Bxe_A1481 of *Burkholderia xenovorans* LB400 (YP_559525; 48% identity and 69% positive matches), BCh11DRAFT_2502 of *Burkholderia* sp. Ch1-1 (ZP_06841236; 48% identity and 69% positive matches), pRi2659_p014 of

Agrobacterium rhizogenes (YP_001960988; 45% identity and 63% positive matches), cucumopine synthase of *Agrobacterium rhizogenes* (BAB13344; 45% identity and 63% positive matches), hypothetical protein of *Agrobacterium rhizogenes* (ABS11829; 45% identity and 64% positive matches) and cucumopine synthase of *Agrobacterium rhizogenes* (CAB65900; 42% identity and 59% positive matches). Among these proteins, two proteins (BAB13344 and CAB65900) have been characterized as cucumopine synthase that catalyzes a coupling between L-histidine and α-ketoglutaric acid by using NADH as a cofactor (Suzuki et al., 2001). However, these proteins have no apparent functional motifs. The predicted 377-aa gene product of *kse_70650* belongs to an FAD-dependent oxidoreductase family with the highest similarity to Caci_2536 of *Catenulispora acidiphila* DSM 44928 (31% identity and 49% positive matches). There are only a few actinomycetes homologues of the KSE_70640 and KSE_70650 proteins with low similarity, and no cluster resembling the region between *kse_70650* and *kse_70630* is found in the current database of the microbial genome.

3.4 Discussion

β-Carboline alkaloids are widely distributed in various plants, whereas only a few reports are available on the β-carboline compounds from bacterial origins (Fotso et al., 2008). This is the first report on the isolation of β-carboline alkaloid from *Kitasatospora* spp. β-Carboline alkaloids are a large group of natural and synthetic indole alkaloids that possess a common tricyclic pyrido[3,4-b] indole ring structure (Cao et al., 2007). Recent interest in these alkaloids has arisen from their diverse biological activities (the potent antitumor, antiviral, antimicrobial, and antiparasitic activities). The structure-activity relationship of β-carboline alkaloids shows that substitutions at C-1 and C-3 in the pyrido ring remarkably affect biological activities, and appropriate substitution could enhance those activities

dramatically, especially antitumor activity (Cao et al., 2007). Although no significant activity of kitasetaline (**2**) was observed under the assays in this study, kitasetaline containing a unique *S*-ethylated *N*-acetylcysteine moiety at C-1 would be an attractive starting compound to design and synthesize novel clinical drugs.

With respect to the biosynthetic pathway of β-carboline alkaloids, strictosidine synthase (STR1) of *Rauvolfia serpentina* (STR1; accession No. P68175), which catalyzes a coupling reaction between tryptamine and secologanin by way of a stereo-selective Pictet-Spengler reaction, is the key enzyme in the biosynthesis of 2,000 indole alkaloids in plants. Our extensive search of the actinobacteria genome database revealed that similar homologues are frequently found to be embedded on the genome of Noca_2860 of *Nocardioides* sp. JS614 (YP_924049; 48% identity and 69 % positive matches), GOARA_019_00250 of *Gordonia araii* NBRC 100433 (ZP_09210674; 30% identity and 46 % positive matches), SSOG_07704 of *Streptomyces hygroscopicus* ATCC 53653 (ZP_07299621; 36% identity and 52% positive matches), SSMG_05546 of *Streptomyces* sp. AA4 (ZP_07281506; 28% identity and 42% positive matches), SSEG_06081 of *Streptomyces sviceus* ATCC 29083 (ZP_06915048; 28% identity and 43% positive matches), and SSQG_00525 of *Streptomyces viridochromogenes* DSM 40736 (ZP_07301638; 28% identity and 42% positive matches), but *K. setae* lacks such orthologue, suggesting that simple computational analysis based on the sequence homology has not been sufficient to find a biosynthetic route of β-carboline compounds in *K. setae*. Here, we successfully identified three biosynthetic genes involved in the biosynthesis of kitasetaline and its derivatives by heterologous expression, although we cannot eliminate the possibility that PabA/B proteins (*p*-aminobenzoate synthases) might be involved in the biosynthesis in addition to these three proteins. The predicted gene product of *kse_70640* with no identifiable motifs resembles cucumopine and mikimopine synthases from *Agrobacterium rhizogenes*. Cucumopine is a stereoisomer of mikimopine, which is

synthesized from L-histidine and α-ketoglutaric acid via formation of Schiff base and its iminium cation by coupling α-amino residue of L-histidine and β-carbonyl residue of α-ketoglutarate (Suzuki et al., 2001). Therefore, the KSE_70640 protein presumably catalyzes a coupling between L-tryptophan and succinate semialdehyde, and the cyclization of the side chain by nucleophilic addition of aromatic ring to iminium cation generated through the Pictet-Spengler reaction to form tryptoline derivative [1-(2-carboxyethyl)-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylic acid]. Currently, an *in vitro* experiment is under way to further clarify the detailed reaction mechanism. Moreover, it is probable that the KSE_70650 protein (putative FAD-dependent oxidoreductase) is involved in the oxidation of piperidine ring in tryptoline derivative to generate β-carboline derivatives. The KSE_70630 protein (putative P450 monooxygenase) would be concerned with the formation of kitasetaline. A significant decrease of the production of kitasetaline and JBIR-133 was observed by deletion of *kse_70700* encoding putative major facilitator superfamily transporter, which often functions as an efflux pump of low molecular-weight compounds. Thus, the KSE_70700 protein is probably a transmembrane-type transporter for the export of kitasetaline and JBIR-133 from the cell.

3.5 Summary

In Chapter 3, we successfully determined the chemical structure of kitasetaline from culture of *K. setae*, and JBIR133 and JBIR134 from heterologous expression of *K. setae* genes in *Streptomyces* host strain, as novel derivatives of β-carboline alkaloids. We, furthermore, determined bioactivity of kitasetaline against methicillin-resistant *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Candida albicans*, nematocidal activity, and effect on leukemic cells, however, no bioactivity could be observed. Moreover,

genes responsible for the biosynthesis of β-carboline could be identified for the first time, leading to the description of plausible route of β-carboline biosynthesis.

Chapter 4

General conclusion

Actinomycetes are gram-positive filamentous bacteria which exhibit two typical features. One feature, not found in other bacteria, is their complex cycle of morphological differentiation, such as germination of spores, elongation and branching of substrate mycelia, formation of aerial mycelia, and septation of hyphae and spore maturation. The second feature is their ability to produce numerous kinds of useful bioactive compounds, including antibiotics, anti-tumor, anti-viral and anti-parasitic compounds, immunosuppressants and so on. About two-thirds of the commercially important antibiotics are produced by bacteria belonging to the genus *Streptomyces* which occupy the major numbers in the group actinomycetes. However, other strains that are called non-*Streptomyces* actinomycetes are also a promising source of medical bioactive compounds and have attracted interest from the viewpoint of providing novel natural products.

The secondary metabolism in actinomycetes is regulated by a variety of physiological and nutritional conditions and is coordinated by morphological development. One of the well known regulatory systems, which controls secondary metabolism and/or morphological differentiation, is the regulation by γ-butyrolactone autoregulators. This system has generally been found in the genus *Streptomyces*. In the absence of the autoregulator, a specific receptor will bind to the promoter region of the target genes and repress their transcription. Produced over the threshold level, the autoregulator will bind to the receptor, resulting in dissociation of the receptor, and thereby triggering transcription of the target gene and allowing the onset of secondary metabolism and/or morphological development. The existence of an autoregulator and its receptor was also previously reported in *Kitasatospora setae*. In this

case, the first autoregulator receptor homologue from a non-*Streptomyces* actinomycete, KsbA, was clarified for its function as the negative regulator of bafilomycin production. However, since the genome sequence of *K. setae* has been made available, we have been able to identify two other homologues of KsbA in the genome, KsbB and KsbC. From the intriguing characteristics and homology of their sequences to autoregulator receptors, together with the presence of *afsA*-family genes in the proximal region, it was suggested that these two receptors might be involved in the regulation of secondary metabolism in *K. setae*. We then aimed to clarify the regulatory function of KsbC.

This thesis is divided into 4 chapters. The preceding chapters have been organized as the follows. The basic knowledge related to this study was shown in Chapter 1. The general introduction explained what actinomycetes are and how they are attractive from the perspective of the production of useful secondary metabolites. In addition, information on the model strain in this study, *Kitasatospora setae*, was provided along with its genome information. The well-known γ-butyrolactone autoregulator signaling cascade, including the example of autoregulators and their receptors, was summarized. Lastly, the details of β-carboline alkaloids together with their biological activities were explained.

In Chapter 2, I reported on the pleiotropic control of morphological development and secondary metabolism by KsbC in *K. setae*. Phenotypic analysis demonstrated that KsbC positively controls bafilomycin production and aerial mycelium formation. Unlike in the case of KsbA, KsbC acts as a negative regulator of kitasetaline and its derivatives production. Choi et al. (2004) revealed the existence of KsbA in *K. setae*, as the first autoregulator receptor homologue in a non-*Streptomyces* actinomycete. KsbA negatively controls bafilomycin production but not morphological development, which is different from its homologue, KsbC. In addition, we found no effect of *ksbA* gene disruption on kitasetaline

production. These findings indicated that KsbC is the second autoregulator receptor in *K. setae* that has pleiotropic regulatory functions on secondary metabolism and morphological differentiation. Interestingly, the results of the transcriptional analysis showed that KsbA and KsbC do not control one another's transcription as well as *bfmR* transcription. It is suggested that there are at least two pathways regulating bafilomycin production. In addition, KsbC are shown to control *ksbS4* transcription. The regulation of ksbS4 transcription by KsbC is opposite to previous understanding that usually receptor will repress the gene transcription, resulting in latter regulation. These findings are suggesting more diverse of regulation which might be particular for each metabolisms.

Chapter 3 presents the completion of the isolation and structure elucidation of kitasetaline, JBIR133 and JBIR134, novel β-carboline derivatives, all of which were discovered for the first time in *K. setae*. It is noteworthy that kitasetaline production was dramatically increased in the *ksbC* disruptant (Chapter 2), making it possible to detect and purify the compound. Furthermore, the results of the heterologous expression of genes from *K. setae* into the *S. avermitilis* SUKA22 host strain provided us a BAC clone harboring biosynthetic genes for kitasetaline production. The identification of genes for kitasetaline biosynthesis revealed the plausible roles of three genes, *kse_70630-kse_70650*, in catalyzing the β-carboline formation, which is the first report of biosynthetic genes of β-carboline in bacteria. According to the biological activities, an appropriate substitution in the position C-1 or C-3 of the β-carboline structure remarkably affects the activities. Although kitasetaline exhibited no bioactivity against tested assays, kitasetaline containing a unique *S*-ethylated *N*-acetylcysteine moiety at C-1 would be an attractive starting compound for the design and synthesis of novel clinical drugs.

In this thesis, I characterized the regulatory function of KsbC, a second autoregulator receptor in *K. setae*, suggesting the pleiotropic control of two typical features by KsbC. From

the discovery of kitasetaline, a novel β-carboline alkaloid, it is suggested that by manipulation of the autoregulator receptor gene we would be able to manipulate the secondary metabolism, which could lead to the discovery of novel natural products in actinomycetes, especially in rare actinomycetes. Since there are still many genes and gene clusters of unknown function as well as autoregulator receptor homologues not only in *K. setae* but also in other actinomycetes, clarifying the function of the genes involved in secondary metabolism will reveal a more diverse array of regulatory cascades utilizing autoregulators. Moreover, from identification of biosynthetic genes for β-carboline compound for the first time in this study, genetic engineering by utilizing this gene information could also be a promising approach to synthesize a various kinds of β-carboline alkaloid and discover a new bioactivity from the new β-carboline derivatives.

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Related publications

1. **Aroonsri, A., Kitani, S., Ikeda, H. & Nihira, T. (2012).** Kitasetaline, a novel β-carboline alkaloid from *Kitasatospora setae* NBRC 14216 ^{T}. *J Biosci Bioeng* **114**, 56–58.
2. **Aroonsri, A., Kitani, S., Hashimoto, J., Kosone, I., Izumikawa, M., Komatsu, M., Fujita, N., Takahashi, Y., Shin-ya, K., Ikeda, H. & Nihira, T. (2012).** Pleiotropic control of secondary metabolism and morphological development by KsbC, a butyrolactone-autoregulator receptor homologue in *Kitasatospora setae*. *Appl Environ Microbiol* (In press).

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