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Goadsporin, a Chemical Substance which Promotes Secondary Metabolism and Morphogenesis in Streptomycetes

I. Purification and Characterization

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Streptomycetes, which belong to the Gram-positive bacteria, produce secondary metabolites and sporulate. The timing of starting the secondary metabolite production and the sporulation depends on environmental conditions such as nitrogen and carbon sources. In order to obtain a tool for understanding the regulation mechanism, we carried out screening for chemical substances that induce secondary metabolism and sporulation in streptomycetes and found an active substance from the culture broth of *Streptomyces* sp. TP-A0584. This substance designated goadsporin promoted the formation of red pigment and sporulation at a concentration of 1 μM in *Streptomyces lividans* TK23 which does not produce the pigment under normal growth conditions. Goadsporin is an oligopeptide consisting of 19 amino acids with the molecular formula $C_{72}H_{97}N_{19}O_{20}S_2$. Sporulation and/or secondary metabolite production was induced in 36 streptomycetes strains among 42 strains tested. These results suggest that goadsporin acts on a common regulation pathway for sporulation and secondary metabolism in streptomycetes and can be a powerful tool to analyze the regulation mechanism.

A number of antibiotics and other bioactive molecules with a variety of chemical structures have been found from actinomycetes as their secondary metabolites. Owing to this diversity, extensive screening for bioactive compounds from actinomycetes has been conducted, aiming at pharmaceutical usage. In screening of soil-derived actinomycetes, it is widely recognized that growth conditions influence the production of secondary metabolites because actinomycetes regulate secondary metabolism and morphogenesis in response to environmental conditions. In industrial fermentation, physical and chemical conditions such as temperature, aeration and medium composition are strictly controlled to gain the maximum productivity.

In recent years, molecular microbiological studies have disclosed common regulation systems for secondary metabolism and cell differentiation in streptomycetes[1,2]. For example, in *Streptomyces griseus*, *Streptomyces virginiae* and *Streptomyces coelicolor* A3(2), low molecular-weight substances containing a butyrolactone serve as chemical signal molecules or microbial hormones for secondary metabolism and/or cellular differentiation[3,4]. In addition, eukaryotic protein kinases[5] and sigma cascades[6] are shown to exist as common components in the regulatory pathway.

S. lividans is known to possess a complete set of the biosynthetic genes for actinorhodin, a red pigment, although it dose not produce the pigment under conventional growth conditions. Interestingly, it is reported that the *afsR* gene that was cloned from *S. coelicolor* A3(2) stimulates actinorhodin production in *S. lividans*[7]. This result shows that *S. lividans* has the potential to produce

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actinorhodin under specific conditions while the production is repressed under usual conditions.

There are several reports on small molecules that induce the secondary metabolism and/or cell differentiation in streptomycetes. A-factor, the most well known butyrolactone, induces streptomycin production, streptomycin resistance, yellow pigment production and aerial mycelium formation at a concentration of less than 1 nM in *S. griseus*[8~10]. Virginia butanolide and IM-2, other butyrolactones, induce the production of virginiamycin in *S. virginiae*, and those of showdomycin and minimycin in *S. lavendulae*, respectively[11,12]. Recently, homologues of the A-factor receptor gene were cloned from *S. coelicolor* A3(2) and shown to be involved in pigment production and cell differentiation[13]. These findings suggest that small molecules containing a butyrolactone moiety are widely distributed in streptomycetes as microbial hormones. Their hormone-like activity is observed only against the hormone-producing strain itself but not against the non-producing streptomycetes, indicating a high specificity in the recognition of the butyrolactones. Pamamycin-607, another signal molecule in streptomycetes, possesses a sixteen-membered macrodiolide structure and was isolated from *Streptomyces alboniger* IFO 12738[14]. This molecule induces aerial mycelium formation at 0.1 μg/paper disc in its producing strain, but the activity is observed only with the producing strain.

To date there is no report on substances that induce secondary metabolism and morphogenesis in a wide range of *Streptomyces* species although the presence of a common regulatory system is suggested in streptomycetes. In this paper, our successful screening for such inducer from soil-derived actinomycetes, and the purification, characterization and biological activity of the novel inducer, goadsporin, are described.

Materials and Methods

Media and Strains

The production culture for actinomycetes was A-3M medium, consisting of glucose 0.5%, glycerol 2.0%, soluble starch 2.0%, pharmamedia 1.5%, Yeast extract (Difco Laboratory, MI) 0.3% and Diaion HP-20 1.0% (pH 7.0). The seed culture for actinomycetes was V-22 medium, consisting of soluble starch 1.0%, glucose 0.5%, NZ-case 0.3%, Yeast extract (Difco) 0.2%, Bacto Tryptone 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). Bennett's agar and Nutrient Broth agar medium were used for the paper disc diffusion assay. Bennett's agar consisted of Yeast extract (Difco) 0.1%, Meat extract (Wako Pure Chemicals) 0.1%, NZ amine 0.2%, glucose 1.0% (pH 7.2). Nutrient Broth agar was purchased from Difco Lab.

Bacterial strains used in this study are shown in Table 1. ATCC strains were obtained from the American Type Culture Collection, Rockville, MD. IFO strains were obtained from the Institution of Fermentation, Osaka, Japan. A NRRL strain was obtained from the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, IL.

Sample Preparation for Paper Disc Diffusion Assay

Actinomycete strains isolated from soil samples collected in Kosugi-machi, Toyama prefecture, Japan, were used for screening. The isolated strains were inoculated into 500-ml K-1 flasks containing 100 ml of V-22 medium. After incubation at 30°C for 4 days on a rotary shaker at 200 rpm, 5-ml aliquots of this seed culture were transferred into 500-ml K-1 flasks, each containing 100 ml of A-3M medium. The fermentation was carried out at 30°C for 7 days on the same rotary shaker. The culture broth was extracted with 100 ml of *n*-butanol, and the extract was subjected to the screening assay.

Assay Procedure for Secondary Metabolism and Sporulation Promoter

Nutrient broth soft agar containing 10^8 spores of *S. lividans* TK23 was overlaid on Bennett's agar plates. *n*-Butanol extracts described above were absorbed in paper discs (diameter: 10 mm), and the discs dried, placed on the plates and incubated at 30°C. Pigment production and cell differentiation around the paper disc were observed every 24 hours. Spore suspension was prepared according to the procedure reported by D. A. HOPWOOD *et al.*[15].

Purification of Goadsporin

The paper disc diffusion assay described above was used to detect the active fractions. The amount and purity of goadsporin was estimated by HPLC analysis as described below.

(i) Fermentation: *Streptomyces* sp. TP-A0584 was inoculated into 500-ml K-1 flasks containing 100 ml of V-22 medium. After incubation at 30°C for 2 days on a rotary shaker at 200 rpm, 5-ml aliquots of the seed culture were transferred into one hundred 500-ml K-1 flasks, each containing 100 ml of A-3M medium. The fermentation was carried out at 30°C for 4 days.

(ii) Diaion HP-20 column chromatography: The cultured broth (10 liters) was centrifuged at 5000×*g* for 10

Table 1. Bacterial strains.

Designation	Relevant characteristics	Source of reference
Streptomyces sp. TP-A0584	goadsporin producer	this study
for goadsporin assay		
S. lividans TK23	goadsporin responder	D. A. Hopwood
S. spinicoumarensis ATCC29813		ATCC
S. violaceoruber IFO15146		IFO
S. griseolus NRRL3739		NRRL
S. hygroscopicus TP-A0342		this study
Microbispora sp. TP-A0184		21
Streptomyces sp. TP-A0585		this study
Streptomyces sp. TP-A0586		this study
Streptomyces sp. TP-A0587		this study
Streptomyces sp. TP-A0588		this study
Streptomyces sp. TP-A0589		this study
Streptomyces sp. TP-A0590		this study
Streptomyces sp. TP-A0591		this study
strain TP-A0593	unidentified actinomycete	this study
for antibiotic and antifungal assay		
Streptomyces scabies JCM7914	Gram(+)	JCM
Bacillus subtilis ATCC6633	Gram(+)	ATCC
Escherichia coli NIHJ JC-2	Gram(-)	
Staphhylococcus aureus 209P JC-1	Gram(-)	
Pseudomonas aeruginosa TP-B0270	Gram(-)	this study
Proteus mirabilis ATCC21100	Gram(-)	ATCC
Sacchromyces cerevisiae TP-F0176	yeast cell	this study
Candida albicans TP-F0594	yeast cell	this study
Cryptococcus neoformans ATCC90112	yeast cell	ATCC
Torulopsis glabrata IFO0622	yeast cell	IFO
Aspergillus fumigatus IFO8866	filamentous fungi	IFO

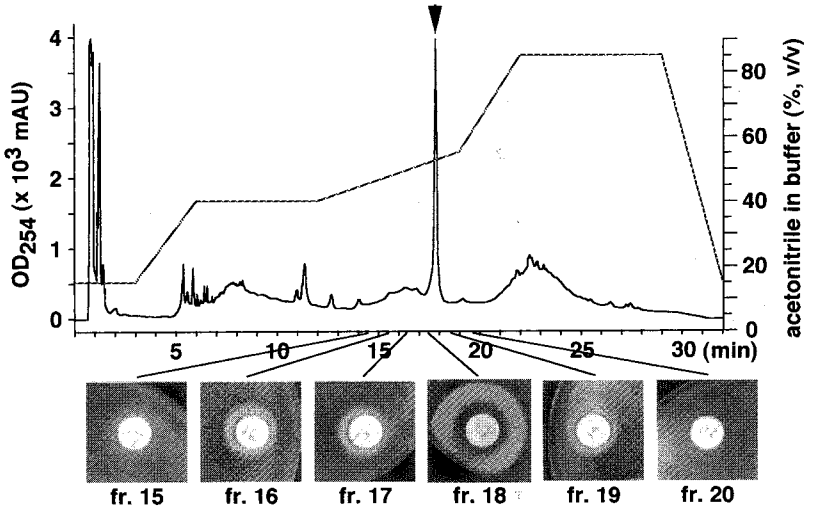
minutes. The supernatant was discarded and the mycelial cake was extracted with 5 liters of 80% aqueous acetone solution three times. After the evaporation to remove acetone, the resultant aqueous solution was applied onto a column of Diaion HP-20 resin (290×95 mm, i.d.; Mitsubishi Chemical Co., Japan) equilibrated with distilled water. The column was washed with 2.5 liters of distilled water and eluted with 5 liters each of 20, 40, 60 and 80% aqueous acetone. Inducing activity for pigment and spore formation was found in the fraction eluted with 60% aqueous acetone. The fraction was concentrated *in vacuo* and the resultant 2.2 liters of aqueous layer was extracted with 1.1 liters of ethyl acetate twice. Concentration of organic extract *in vacuo* gave 2.2 g of crude extract.

(iii) Silica gel column chromatography: The crude extract was dissolved in 10 ml of methanol and applied to a column of silica gel 60 (220×40 mm, i.d.; Merck, NJ) equilibrated with chloroform. The column was eluted with a gradient of chloroform - methanol (30 : 1~2 : 1, 1.5 liters). The fractions containing the active component eluted in chloroform - methanol (5 : 1), and were pooled and evaporated *in vacuo* to give crude goadsporin (782 mg).

(iv) LH-20 gel filtration chromatography: The crude goadsporin was dissolved in 10 ml of chloroform - methanol (1 : 1) and applied to a column of Sephadex LH-20 (860×21 mm, i.d.; Amersham Pharmacia Biotech, UK) equilibrated with chloroform - methanol (1 : 1). The column was eluted with chloroform - methanol (1 : 1). The fractions containing goadsporin were pooled and evaporated *in vacuo* to give semi-pure goadsporin (576 mg).

(v) Reverse phase silica gel column chromatography: The residue dissolved with 10 ml of methanol was applied to an ODS-AM reverse phase silica gel column (200× 46 mm, i.d.; YMC, Japan) equilibrated with acetonitrile - 0.15% K_2HPO_4 (pH 3.5) (2 : 8). The column was eluted with acetonitrile - 0.15% K_2HPO_4 (pH 3.5) (1 : 1). The

Fig. 1. HPLC chromatogram of secondary metabolites of *Streptomyces* sp. TP-A0584.



The arrow shows a peak of the active compound. Sample preparation and HPLC condition are described in Materials and Methods. The elution was performed with a linear gradient as indicated on the right-hand scale. The photos show the sporulation induction assay on *S. lividans* for each fraction samples.

fractions containing active material were pooled and evaporated *in vacuo*. The resultant aqueous layer was extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give pure goadsporin (316 mg).

HPLC Analysis

HPLC analysis was performed with a HP1090 (Hewlett Packard, CA) system using a C18 Rainin microsorb column (3 μm, 100×4.6 mm, i.d.; Rainin Instrument Co., MA). The sample was eluted at a flow rate of 1.2 ml/minute with a gradient of acetonitrile - 0.15% KH_2PO_4 (pH 3.5), detecting at 254 nm. The gradient diagram is shown in Fig. 1.

Antimicrobial Assay

The minimum inhibitory concentrations against bacteria and yeasts were determined by the 2-fold serial dilution method in heart infusion broth or Yeast morphology broth (Difco Lab.) as previously described[16].

Color Photographs

Color photographs for this article are on the internet web site: http://www.pu-toyama.ac.jp/BR/hibari/goadsporin.html.

Results and Discussions

Screening for Inducers of Secondary Metabolism and Morphogenesis

Fermentation broths of 405 actinomycetes strains isolated from soil samples were subjected to the induction test for pigment production in *S. lividans* TK23. A spore suspension of *S. lividans* TK23 was mixed with Nutrient Broth soft agar, and *ca.* 10^8 spores were plated on a Bennett's agar plate. Actinomycetes were cultured in A-3M medium which contains Diaion HP-20 resin and the culture broth was extracted with *n*-butanol. Some reports show that the addition of HP-20 results in a increase of secondary metabolites production[17], therefore we always added HP-20 resin to prepare screening samples. The extracts were assayed, and *Streptomyces* sp. TP-A0584 was found to produce a substance that promotes the formation of diffusible red pigment, aerial hyphae and sporulation in *S. lividans* TK23.

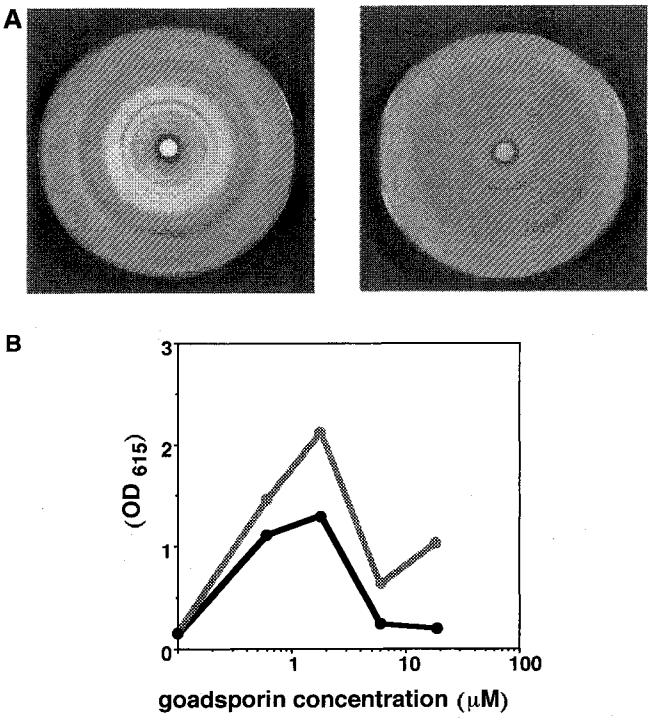
The *n*-butanol extract of *Streptomyces* sp. TP-A0584 was concentrated *in vacuo*, and the residue was fractionated by HPLC. The fractions were assayed in the induction test, and the activity was found to coincide with the peak at 18 minutes (Fig. 1). An extraction test indicated that the active compound is adsorbed or accumulated in the harvested

cells and is not detected in butanol extracts from cell free broth, that is, not released into the culture medium. Its accumulation reaches a maximum in 6 days (data not shown).

Fermentation and Purification of Goadsporin

Production fermentation was carried out for 6 days and the cultured cells were collected by centrifugation. The cells were extracted with an equal volume of 80% aqueous acetone, and the extract was purified by a series of column chromatographies: HP-20 adsorption resin, silica gel, LH-20 gel filtration and reversed phase silica gel. A yield of 316 mg of pure active compound was obtained from 10 liters of culture broth. The purity was confirmed by HPLC and NMR analysis. This compound, designated goadsporin, was named after its ability to stimulate (goad) sporulation. As shown in Fig. 2A (color fig.: http://www.pu-toyama.ac.jp/BR/hibari/fig2.html), ring-shaped zones of sporulation and pigment production were observed around the paper disc containing goadsporin. This figure shows the goadsporin concentration dependency on the precocious induction of sporulation and the red pigment formation. The spore formation in the gray zone was confirmed by electron microscopy analysis (data not shown). Induction of actinorhodin production by goadsporin was examined in Bennett's liquid medium (Fig. 2B). The production of the pigment was observed in the presence of goadsporin but not in its absence and was dose-dependent at less than 1.8 μM. The growth of *S. lividans* was inhibited at the higher concentration. This result agrees with the formation of a ring-shaped zone of pigment and the inhibition zone near the paper disc on the solid agar (Fig. 2A).

Fig. 2. Effect of goadsporin on the morphogenesis and red pigment production of *S. lividans* TK23 on solid medium (A) and on the red pigment production by *S. lividans* TK23 in liquid medium (B).



(A) Photographs were taken after 4 days of growth on Bennett's agar medium. Goadsporin 6 nmol (*ca.* 10 μg), was absorbed on paper discs. Left: a photograph of the surface of the plate. Aerial mycelium formation was observed in the white ring zone, and spore formation was observed in the gray zone inside of the white zone. Right: a photograph of the reverse side of the plate. Red pigment production was observed around the paper disc. (B) Actinorhodin production in Bennett's liquid medium containing various concentrations of goadsporin. Production after 4 days growth (black) and 6 days growth (grey) was determined by following A_{615} of the culture broth at pH 12.

Goadsporin Is a Chemically and Biologically Novel Compound

The structure of goadsporin (Fig. 3) was determined by the analysis of NMR and mass spectra. The molecular formula of goadsporin is $C_{72}H_{97}N_{19}O_{20}S_2$ (monoisotopic MW 1611.66). Goadsporin is a linear oligopeptide containing nineteen amino acids, six of which are cyclized to form four oxazole and two thiazole rings. Most of the amino acids in goadsporin are hydrophobic. The *N*-terminus is acetylated while the *C*-terminus is a free carboxylic acid. A database search of the structure using The Chemical Abstracts file resulted in finding no coincidence with known classes of natural products. The structure determination of goadsporin will be reported in the accompanying paper[18]. The database search found no analogous compounds ever reported, suggesting that goadsporin is a compound specifically active against streptomycetes. In fact, goadsporin did not show any antibiotic activity against non-streptomycetes and no cytotoxic activity, protein kinase C phospholylation inhibition or apoptosis induction in mammalian cells (data not shown). Thus, the molecular target of goadsporin is apparently a specific one in streptomycetes but not common to other organisms such as DNA, protein kinases or ribosomes.

Fig. 3. Structure of goadsporin.

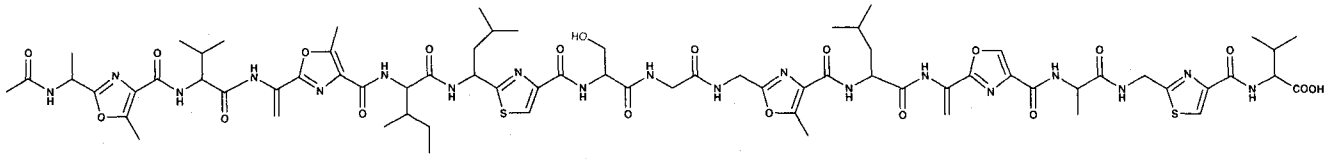
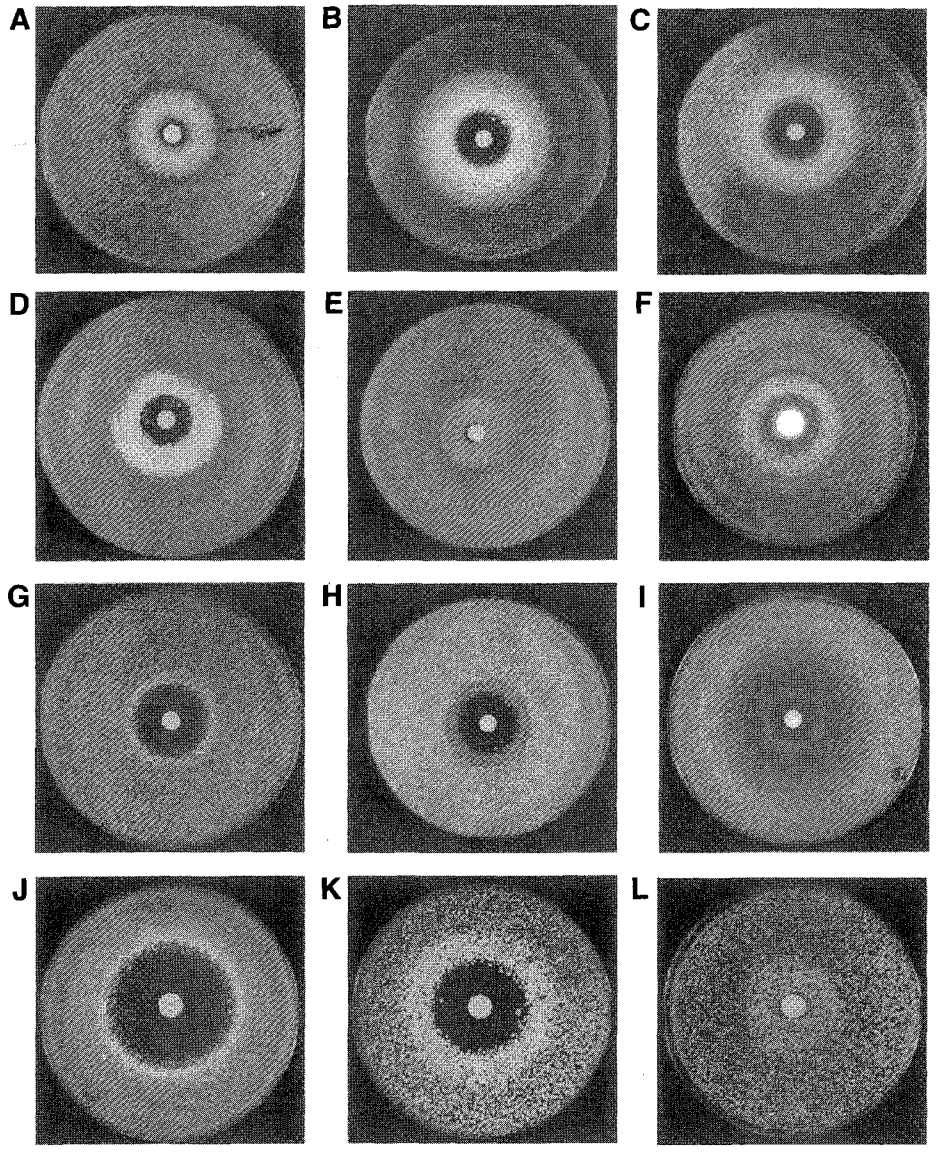


Fig. 4. Effect of goadsporin on morphogenesis and pigment production in various streptomycetes on solid medium using 6 nmol (*ca.* 10 μg) of goadsporin absorbed on paper discs.



Photographs were taken on Bennett's agar medium.

(A) *Streptomyces* sp. TP-A0586, 13 days growth. (B) *Streptomyces* sp. TP-A0588, 7 days. (C) *Streptomyces* sp. TP-A0585, 9 days. (D) *Streptomyces* sp. TP-A0589, 5 days. (E) *Streptomyces* sp. TP-A0590, 5 days. (F) *Streptomyces* sp. TP-A0591, 4 days. (G) *S. spinicoumarensis* ATCC29813, 6 days. (H) *Streptomyces* sp. TP-A0587, 8 days. (I) *S. violaceoruber* IFO15146, 4 days. (J) *S. hygroscopicus* MCRL0401, 10 days. (K) *Microbispora* sp. TP-A0184, 10 days. (L) *S. griseolus* NRRL3739, 10 days.

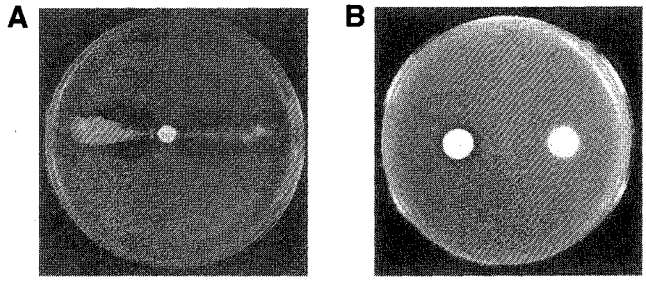
Goadsporin Promotes Morphogenesis in a Wide Variety of Streptomycetes

Induction of secondary metabolism and sporulation was examined with 42 streptomycetes strains randomly selected from the stock strains in our laboratory. Among the 42 strains, at 10 μg/paper disc, sporulation was induced in 32 strains (76%) around the disc (Fig. 4A, B, C, D, F, G, J, K, L or http://www.pu-toyama.ac.jp/BR/hibari/fig4.html), pigment production was promoted in 20 strains (48%) and growth inhibition was observed at the high concentration in 32 strains (76%). It is obvious that the sporulation was induced in some range of goadsporin concentration because the spore formation was observed as a ring-shaped zone around the paper disc. In addition, growth inhibition was caused by goadsporin at high concentration in streptomycetes that respond to goadsporin by sporulating. Since, we expected that some antibiotics might induce sporulation like goadsporin, 200 μg of streptomycin, kanamycin, thiostrepton, bacitracin and gramicidin D were absorbed on paper discs and assayed. However, none of them showed goadsporin-like activity in *S. lividans* TK23. In the life cycle of streptomycetes, formation of the aerial hyphae and the following sporulation takes place after sufficient growth of substrate mycelium. If a high concentration of goadsporin is present in the early stage of the growth, it may promote aerial hyphae formation before enough development of substrate mycelium, resulting in growth inhibition. A recent study revealed that the spore pigment derives from polyketides[19], the most common secondary metabolites in streptomycetes. In this study, we have shown that goadsporin promotes both sporulation and secondary metabolism in several streptomycetes, suggesting the presence of common regulation components for them and the possible involvement of goadsporin.

Goadsporin Promotes Secondary Metabolism in Streptomycetes

Since goadsporin promoted the pigment production in *S. lividans*, it was also expected to induce antibiotic production in other streptomycetes. Thirty eight randomly selected actinomycete strains were tested in the induction assay. The test strains were inoculated in lines on Bennett's agar plates and paper discs containing goadsporin were plated on the centers of the lines. After incubation for several days, NB soft agar containing *Bacillus subtilis* ATCC6633 was overlaid and inhibition zones observed. An unidentified actinomycete TP-A0593 was found to produce an antibiotic against *B. subtilis* around the paper disc (Fig. 5A). In addition, TP-A0593 strain was shown to produce the antibiotic when fermented in Bennett's liquid medium in the presence of 18 μM goadsporin. After cultivation for 8 days, the culture broth was tested using paper disc diffusion assay against *B. subtilis*, and it was confirmed that the production of antibiotic was induced by goadsporin (Fig. 5B).

Fig. 5. Effect of goadsporin on anitibiotic production by an unidentified actinomycete TP-A0593.



(A) A photograph was taken after *Bacillus subtilis* ATCC6633 was overlaid on Bennett's agar medium. Six nmol (*ca.* 10 μg) of goadsporin was absorbed on a paper disc, placed on a line of strain TP-A0593 and incubated 4 days. The inhibition zone was observed near the paper disc depending on amounts of diffused goadsporin. (B) A photograph of the paper disc diffusion assay against *B. subtilis*. Paper discs contained the culture broth of strain TP-A0593 cultivated for 8 days in the presence of 18 μM goadsporin (left) or in its absence (right). An inhibition zone was observed around the paper disc containing the broth cultured in the presence of goadsporin (left).

Antibiotic Activity of Goadsporin

The antibiotic activity of goadsporin was examined (Table 2). It showed growth inhibition against *Streptomyces*, but not against the other microorganisms at 60 μM. *S. scabies*, the cause of potato scab, is often found in potato growing areas and causes considerable economic loss in the world. It should be noted that goadsporin has potential utility as an agrochemical for potato scab.

Goadsporin Does Not Have Any Biological Activity Against Its Producing Strain

Interestingly, goadsporin did not induce the precocious spore and pigment formation in the goadsporin-producing strain, *Streptomyces* sp. TP-A0584, when it was added to

Table 2. *In vitro* antibacterial activities of goadsporin.

Organism	Relevant characteristics	MIC (μg/ml)
Streptomyces lividans TK23	Gram(+)	6.4
Streptomyces coelicolor A3(2)	Gram(+)	3.2
Streptomyces scabies JCM7914	Gram(+)	0.2
Bacillus subtilis ATCC6633	Gram(+)	>100
Escherichia coli NIHJ JC-2	Gram(-)	>100
Staphhylococcus aureus 209P JC-1	Gram(-)	>100
Pseudomonas aeruginosa TP-B0270	Gram(-)	>100
Proteus mirabilis ATCC21100	Gram(-)	>100
Sacchromyces cerevisiae TP-F0176	yeast cell	>100
Candida albicans TP-F0594	yeast cell	>100
Cryptococcus neoformans ATCC90112	yeast cell	>100
Torulopsis glabrata IFO0622	yeast cell	>100
Aspergillus fumigatus IFO8866	filamentous fungi	>100

the medium (data not shown). All of the reported signal molecules in streptomycetes induce sporulation or secondary metabolism in the producing strain. Goadsporin is different from those hormone-like compounds because it does not affect its producing strain. Considering the observation that the producing strain accumulates goadsporin within the cell, goadsporin may not permeate the cell membrane. Some of the tested streptomycetes did not respond to goadsporin stimulation, and this could be for the same reason. Recently UEDA *et al.* reported that there are inter-specific stimulatory events on antibiotic production and sporulation among streptomycetes[20]. One possible function for goadsporin in nature is that *Streptomyces* sp. TP-A0584 controls different *Streptomyces* species using goadsporin for growth inhibition or sporulation acceleration to favor its survival. However, the producing strain may fail to use goadsporin for this purpose in nature because of the lack of membrane permeation.

Goadsporin Is a Useful Tool for Analyzing the Regulation of Secondary Metabolism and Morphogenesis

We obtained some mutants insensitive to goadsporin from *S. lividans* TK23 by mutagenesis in order to study the mode of action of goadsporin. These mutants show no response to goadsporin but maintain a potential to produce the red pigment and to sporulate as well as the parent strain. Cloning of the responsible genes is in progress. Further study is expected to elucidate not only the mechanism of goadsporin but also the regulation of sporulation and secondary metabolism in streptomycetes.

Acknowledgments

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References

1) BIBB, M.: The regulation of antibiotic production in *Streptomyces coelicolor* A3(2). Microbiol. 142: 1335~1344, 1996
2) CHATER, K. F.: Taking a genetic scalpel to the *Strepomyces* colony. Microbiol. 144: 1465~1478, 1998
3) HORINOUCHI, S. & T. BEPPU: Autoregulatory factors and communication in actinomycetes. Annu. Rev. Microbiol. 46: 377~398, 1992
4) YAMADA, Y.: Butyrolactone autoregulators, inducers of secondary metabolites, in *Streptomyces*. Actinomycetol. 9: 57~65, 1995
5) MATSUMOTO, A.; S. K. HONG, H. ISHIZUKA, S. HORINOUCHI & T. BEPPU: Phosphorylation of the AfsR protein involved in secondary metabolism in *Streptomyces* species by a eukaryotic-type protein kinase. Gene 146: 47~56, 1994
6) CHATER, K. F.; C. J. BRUTON, K. A. PLASKITT, M. J. BUTTNER, C. MENDEZ & J. D. HELMANN: The developmental fate of *S. coelicolor* hyphae depends upon a gene product homologous with the motility sigma factor of *B. subtilis*. Cell 59: 133~143, 1989
7) HORINOUCHI, S. & T. BEPPU: Production in large quantities of actionorhodin and undecylprodigiosin induced by *afsB* in *Streptomyces lividans*. Agric. Biol. Chem. 48: 2131~2133, 1984
8) HORINOUCHI, S. & T. BEPPU: A-factor as a microbial hormone that controls cellular differentiation and

secondary metabolism in *Streptomyces griseus*. Mol. Microbiol. 12: 859~864, 1994

9) KHOKHLOV, A. S.; I. I. TOVAROVA, L. N. BORISOVA, S. A. PLINER, L. A. SCHEVCHENKO, E. Y. KORNITSKAYA, N. S. IVKINA & I. A. RAPOPORT: A-factor responsible for the biosynthesis of streptomycin by a mutant strain of *Actinomyces streptomycini*. Dokl. Akad. Nauk SSSR 177: 232~235, 1967

10) ONAKA, H.; N. ANDO, T. NIHIRA, Y. YAMADA, T. BEPPU & S. HORINOUCHI: Cloning and characterization of the A-factor receptor gene from *Streptomyces griseus*. J. Bacteriol. 177: 6083~6092, 1995

11) SATO, K.; T. NIHIRA, S. SAKUDA, M. YANAGIMITO & Y. YAMADA: Isolation and structure of a new butyrolactone autoregulator from *Streptomyces* FRI-5. J. Ferm. Bioeng. 68:170~173, 1989

12) YAMADA, Y.; K. SUGAMURA, K. KONDO, M. YANAGIMOTO & H. OKADA: The structure of inducing factors for virginiamycin production in *Streptomyces virginiae*. J. Antibiotics 40: 496~504, 1987

13) ONAKA, H.; T. NAKAGAWA & S. HORINOUCHI: Involvement of two A-factor receptor homologues in *Streptomyces coelicolor* A3(2) in the regulation of secondary metabolism and morphogenesis. Mol. Microbiol. 28: 743~753, 1998

14) KONDO, S.; K. YASUI, M. NATSUME, M. KATAYAMA & S. MARUMO: Isolation, physico-chemical properties and biological activity of pamamycin-607, an aerial mycelium-inducing substance from *Streptomyces alboniger*. J. Antibiotics 41: 1196~1204, 1988

15) HOPWOOD, D. A.; M. J. BIBB, K. F. CHATER, T. KIESER, C. J. BRUTON, H. M. KIESER, D. J. LYDIATE, C. P. SMITH, J. M. WARD & H. SCHREMPF: Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom., 1985

16) OTANI, T.; Y. SUGIMOTO, Y. AOYAGI, Y. IGARASHI, T. FURUMAI, N. SAITO, Y. YAMADA, T. ASAO & T. OKI: New Cdc25B tyrosine phosphatase inhibitors, Nocardiones A and B, produced by *Nocardia* sp. TP-A0248: Taxonomy, fermentation, isolation, structural elucidation and biological properties. J. Antibiotics 53: 337~344, 2000

17) HARA, M.; T. MOKUDAI, E. KOBAYASHI, K. GOMI & H. NAKANO: The kapurimycins, new antitumor antibiotics produced by *Streptomyces*. J. Antibiotics 43: 1513~1518, 1990

18) IGARASHI, Y.; Y. KAN, K. FUJII, T. FUJITA, K. HARADA, H. NAOKI, H. TABATA, H. ONAKA & T. FURUMAI: Goadsporin, chemical substance which promotes secondary metabolism and morphogenesis in streptomycetes. II. Structure determination. J. Antibiotics 54: 1045~1053, 2001

19) DAVIS, N. K. & K. F. CHATER: Spore colour in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. Mol. Microbiol. 4: 1679~1691, 1991

20) UEDA, K.; S. KAWAI, H. OGAWA, A. KIYAMA, T. KUBOTA, H. KAWANOBE & T. BEPPU: Wide distribution of interspecific stimulatory events on antibiotic production and sporulation among *Streptomyces* species. J. Antibiotics 53: 979~982, 2000

21) IGARASHI, Y.; K. TAKAGI, T. KAJIURA & T. FURUMAI: Gulycosylquestiomycin, a novel antibiotic from *Microbispora* sp. TP-A0184: fermentation, isolation, structure determination, synthesis and biological activities. J. Antibiotics 51: 915~920, 1998