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COMMUNICATIONS TO THE EDITOR

Wide Distribution of Interspecific Stimulatory Events on Antibiotic Production and Sporulation Among *Streptomyces* species

Sir:

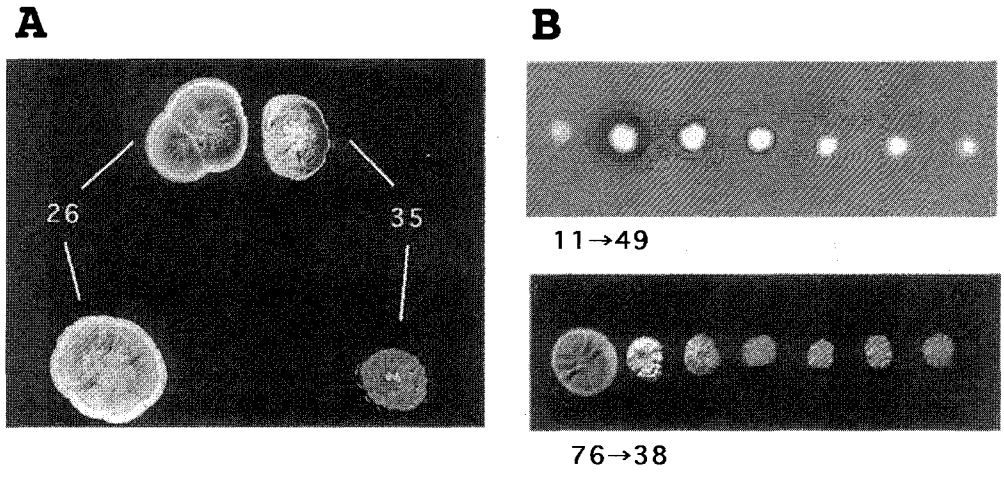
Diffusible autoregulatory factors such as A-factor and virginiae butanolides (VB) are known to stimulate antibiotic production and/or aerial mycelium formation in a variety of *Streptomyces* species[1~3]. Most of these factors which contain a γ-butyrolactone ring have been discovered by intraspecific feeding experiments, in which an extract of the culture supernatant of one species stimulated antibiotic production and/or cellular differentiation of the same species. The regulatory mechanisms by which these autoregulatory factors function have been precisely studied at a molecular level, and it is suggested that the strict ligand-specificity of their receptors confine the activity of the factors to species-specific self-regulation[1,2]. There is, however, another possible consequence if the species-specificity of diffusible factors is low—it would enable communication between different *Streptomyces* species that shared common signal substances. We have conducted comprehensive pairing analyses with *Streptomyces* to examine the possibility of interspecific stimulation of antibiotic production and/or sporulation.

The first analysis was done with 76 strains chosen from a culture collection (Table 1). Each pair of strains was inoculated on a Bennett's-glucose agar plate to form single colonies either close (1 cm) or furthermost (5 cm) from each other (see Fig. 1A). Stimulation of aerial mycelium formation at close range was frequently checked during cultivation at 28°C. After an appropriate period (1~3 days), plates were overlaid with soft agar containing spores of *Bacillus subtilis* ATCC6633 and incubated overnight at 37°C. Antibiotic activity was estimated by the appearance

Table 1. Strains from culture collection.

No.	strain	No.	strain	No.	strain
1.	*S. salmonicida* NRRL B-1472	26.	*S. flavochromogenes* IAM0059	51.	*Streptomyces* sp. st-20-8
2.	*S. tanashiensis* IAM0041	27.	*S. thioluteus* st-16-b	52.	*Streptomyces* sp. st-20-5
3.	*S. fradiae* IFO3123	28.	*S. sindenensis* IFO1071	53.	*Streptomyces* sp. st-19-7
4.	*S. rimosus* IAM0014	29.	*S. bobiliae* IAM0002	54.	*S. zaomyceticus* st-25-11
5.	*S. viridochromogenes* st-24-1	30.	*S. phaeochromogenes* st-24-5	55.	*S. flaveolus* IAM0117
6.	*S. phaeochromogenes* IAM0038	31.	*S. lavendulae* Ru3340-8	56.	*Streptomyces* sp. st-20-2
7.	*S. roseochromogenes* IAM0039	32.	*S. halstedii* IFO3197	57.	*S. cinnamonensis* IAM0020
8.	*S. viridochromogenes* IFO3113	33.	*Streptomyces* sp. st-19-8	58.	*S. viridochromogenes* st-18-a
9.	*S. parvulus* IAM0062	34.	*S. purpurascens* IAM0052	59.	*S. viridochromogenes* st-24-3
10.	*S. tanashiensis* IAM0016	35.	*Streptomyces* sp. st-19-6	60.	*S. griseolus* IFO587
11.	*S. californicus* IAM0003	36.	*S. scabies* IAM0015	61.	*S. fradiae* Ru3535
12.	*S. griseus* Ru3463	37.	*S. coelicolor* IAM1023	62.	*S. rubescens* IAM0074
13.	*S. albogriseolus* IAM0031	38.	*S. mitakaensis* st-25-3	63.	*S. antibioticus* NRRL B-546
14.	*S. roseochromogenes* st-14-b	39.	*S. flavovirens* IAM0069	64.	*S. roseochromogenes* st-23-6
15.	*S. erythraeus* IAM0045	40.	*S. tanashiensis* st-25-4	65.	*S. albus* IAM0067
16.	*S. olivaceus* NRRL B-1125	41.	*S. coelicolor* st-22-3	66.	*S. coelicolor* st-22-2
17.	*S. aureus* IAM0092	42.	*Streptomyces* sp. st-19-9	67.	*S. albus* NRRL B-3381
18.	*S. sulphureus* st-15-c	43.	*Streptomyces* sp. st-19-5	68.	*S. griseus* st-21-2
19.	*S. rutgersensis* IAM0085	44.	*S. bikiniensis* IAM0019	69.	*S. blastmyceticus* st-24-6
20.	*S. parvus* IAM0013	45.	*S. flavovirens* IAM0077	70.	*S. salmonicida* NRRL B-1474
21.	*S. salmonicida* IAM0054	46.	*S. viridochromogenes* IAM0066	71.	*S. flaveolus* IAM0033
22.	*S. albus* IAM0057	47.	*S. hiroshimensis* st-24-10	72.	*Streptomyces* sp. st-19-1
23.	*S. roseochromogenes* IAM0027	48.	*S. erythraeus* st-22-7	73.	*Streptomyces* sp. st-20-4
24.	*S. viridochromogenes* IAM0086	49.	*S. sulphureus* st-21-4	74.	*S. flaveolus* IAM0046
25.	*S. coelicolor* IAM9023	50.	*Streptomyces* sp. st-19-3	75.	*S. albus* NRRL B-3381
				76.	*S. abikoensis* IAM0001

Fig. 1. Cross-stimulation of antibiotic production and/or sporulation among the 76 strains from culture collection.



C

		stimulator																								
		2	6	7	8	10	11	16	17	21	23	26	29	31	32	36	38	39	40	41	44	49	51	55	68	71	76
receiver	6	a	/	-	a	-	-	a	a	-	-	-	-	a	-	a	a	-	-	-	a	a	-	a	a	-	-
	8	-	-	-	/	-	-	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	24	-	b	b	-	-	-	c	-	-	-	-	a	a	b	b	a	c	-	a	-	-	b	a	a	-	-
	31	-	-	a	-	-	-	a	a	-	-	-	-	-	a	-	a	-	-	a	-	a	a	-	-	-	-
	35	b	b	-	c	a	b	b	b	b	b	b	b	c	c	b	b	b	b	-	b	b	b	b	b	b	b
	36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	a	-	-	-	-	-	-
	38	c	c	-	c	-	c	-	-	-	c	c	-	c	c	-	/	c	c	c	-	c	c	-	c	c	c
	44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	a	-	-	-	-	-	-	-	-	-	-	-
	49	-	-	-	a	-	a	-	a	-	-	-	-	-	-	a	a	a	-	-	a	/	-	a	-	-	-
	55	-	-	-	c	-	-	c	c	-	-	-	c	-	c	-	-	c	-	-	-	c	-	/	c	-	-
	63	a	-	a	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	a	-	-
	65	-	c	-	-	-	-	-	-	-	-	-	-	-	-	c	-	-	-	-	-	-	-	-	-	-	-
	66	-	-	-	b	c	b	c	c	-	c	c	-	c	-	c	c	-	-	a	c	c	-	-	c	-	c
	68	-	-	-	a	-	a	-	a	-	-	-	-	-	-	a	-	-	-	-	-	-	-	a	/	-	-

(A) An example of positive response in the first step pairing analysis. A stimulator strain (No. 26) enhanced sporulation of a receiver strain (No. 35) at the close proximity (upper). (B) Examples of the positive responses in the cross-feeding assay. Stimulator strains No. 11 and No. 76 enhanced antibiotic production of No. 49 and sporulation of No. 38, respectively. (C) Summarised results of cross-feeding assay. Pairs are marked showing interspecific stimulation of; *a*, antibiotic production; *b*, antibiotic production and sporulation; *c*, sporulation.

of growth inhibitory zones. Strains stimulating (stimulator) or being stimulated (receiver) were thus identified and were further subjected to cross-feeding assays to confirm the activities: 6 colonies of a receiver strain were formed in a line at intervals of 1 cm on a Bennett's-glucose agar plate from a colony of a stimulator strain and cultured at 28°C. In these ways the presence of a putative stimulatory factor secreted from a stimulator strain was detected by the induction of antibiotic production or sporulation of the receiver strain in a diffusion-gradient-dependent manner (see Fig. 1B and 2B). As shown in Fig. 1C, 26 among 76 strains (34%) were active in stimulating antibiotic production of 10 strains (13%) or sporulation of 7 strains (9%). Representative results of the cross-feeding assay are shown in Fig. 1B. The stimulator strains did not cause distinct pH changes in the agar plates, which excludes the possibility that stimulation was a result of nonspecific effect of increased pH. Neither 50 nmol of synthetic A-factor, VB-C[2] nor γ-nonalactone showed stimulatory effects on the receiver strains (data not shown).

It was considered possible that the unexpectedly high frequency of cross-activation found in this study was due to

the fact that genetic variations in strains taken from a culture collection. Therefore we carried out a similar analysis with fresh isolates. A cell suspension prepared from a soil sample collected from a field in Fujisawa-city, Kanagawa, Japan, was plated on Bennett's-glucose agar plates and 33 *Streptomyces* (or actinomycete) different strains were isolated according to macroscopic and microscopic observation. As described above, all pairs among the 33 strains were subjected to the cross-stimulation assay. The result is summarised in Fig. 2A; 32 out of 33 strains (97%) showed activities stimulating antibiotic production of 11 strains (33%) or sporulation of 19 strains (58%). Representative results are shown in Fig. 2B. Again none of the strains caused significant pH changes in the medium. A-factor-producing *Streptomyces griseus* IFO13350 showed no stimulatory activity to the receiver strains, which indicates that none of stimulatory substances is A-factor. Furthermore, an experiment with 46 fresh isolates from a different soil sample gave a similar result, *i.e.*, 45 strains (98%) stimulated antibiotic production of 19 strains (41%) or sporulation of 26 strains (57%).

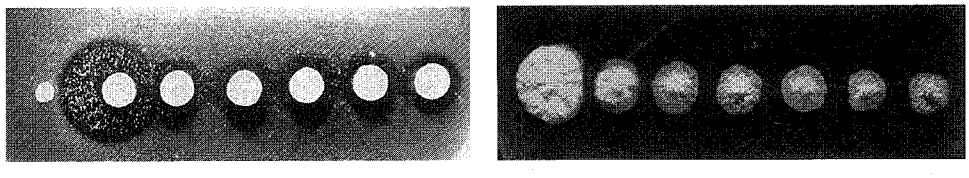
All the results strongly suggest that diffusible substances are involved in physiological stimulation between different *Streptomyces* strains. Preliminary experiments with active strains from the culture collection showed that the active substances of 4 stimulator strains (No. 2, 7, 11 and 17 in

Fig. 2. Cross-stimulation of antibiotic production and/or sporulation among the 33 fresh isolates.

A

receiver \ stimulator	1	2	3	4	5	6	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	/	-	-	-	-	-	-	-	-	-	-	-	-	c	b	c	c	a	-	-	-	-	-	-	-	c	-	-	-	-	-	-
2	-	/	-	-	-	-	-	-	-	-	-	-	-	a	-	-	-	-	-	a	-	a	-	-	-	-	-	-	-	-	-	-
3	-	-	/	-	-	-	-	-	-	-	-	-	-	-	a	-	a	-	-	c	-	-	-	c	-	-	-	-	-	-	-	-
4	-	-	-	/	c	c	-	c	c	c	c	c	-	c	c	-	c	-	c	-	-	-	c	-	-	-	c	-	c	-	c	-
5	-	-	-	-	/	-	-	-	c	-	-	c	-	-	-	-	-	-	c	c	-	-	-	c	-	-	-	-	c	-	-	c
6	-	-	-	-	-	/	-	-	-	-	c	c	-	c	c	c	b	c	c	c	-	-	-	-	-	-	c	-	c	c	-	-
7	-	-	-	c	-	-	/	c	c	c	c	c	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	c
8	-	-	-	-	-	-	-	/	c	c	-	c	c	-	-	-	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	c	c	-	-	c	/	-	c	c	c	c	c	-	-	c	-	c	c	c	c	c	-	-	c	-	c	c	c	c
11	-	-	c	c	-	-	c	c	-	/	c	c	c	c	c	c	-	c	-	c	c	c	c	c	-	-	c	c	c	c	c	c
12	-	-	-	-	a	a	-	-	-	-	/	-	-	-	-	-	a	-	-	-	-	a	a	-	-	-	-	-	-	-	-	-
13	-	-	-	c	c	c	-	c	-	-	-	/	-	c	-	-	-	c	-	-	-	c	-	-	-	-	c	-	-	-	c	-
14	c	c	-	-	-	c	-	-	c	-	c	-	/	c	-	-	-	c	-	-	-	c	c	c	-	-	c	-	c	-	-	-
16	-	-	-	-	a	-	-	-	-	-	-	-	-	/	a	-	-	-	-	-	-	a	-	-	-	-	-	-	-	-	-	-
19	-	-	c	c	-	-	-	-	c	-	-	c	c	-	-	a	-	/	a	a	-	c	-	a	-	-	-	-	-	-	-	c
20	-	-	-	a	-	-	-	-	-	-	-	-	-	-	-	-	-	a	/	-	-	a	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	-	-	-	-	-	a	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	-	-	-	-	-	a	-	-	-	-	-
23	-	-	-	-	-	c	-	-	c	-	c	-	-	-	-	c	-	-	-	c	-	/	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	c	-	-	-	-	-	/	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	c	c	-	c	c	-	c	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-	c	-	-	-	-	-	-	-	-	-	c	-	-	-	-	/	-	-	-
31	-	a	-	-	-	-	a	-	-	-	-	-	a	a	-	-	-	-	-	c	a	-	-	-	-	-	a	-	-	/	-	-
33	-	-	-	-	c	-	-	-	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/

B



6 → 12 25 → 30

(A) Summarised results of cross-feeding assay. Pairs are marked showing interspecific stimulation of; *a*, antibiotic production; *b*, antibiotic production and sporulation; *c*, sporulation. (B) Eamples of the positive responses. Stimulator strains No. 6 and No. 25 enhanced antibiotic production of No. 12 and sporulation of No. 30, respectively.

Fig. 3. Stimulation of antibiotic production by ethyl acetate extracts.

Paper discs containing the extracts of culture supernatant of stimulator strains No. 7 and No. 17 were assayed against receiver strains No. 63 and No. 49, respectively.

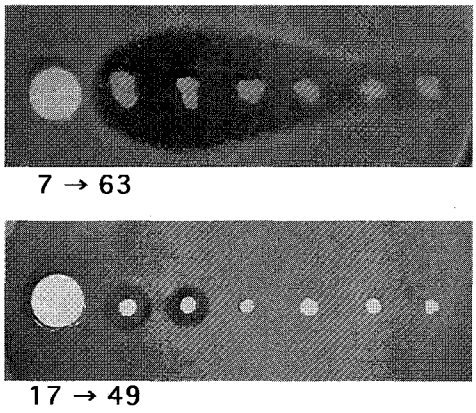


Table 1) were present in the culture broth and were extractable with ethyl acetate. The ethyl acetate extracts from the culture broth of No. 7 (*Streptomyces roseochromogenes*) and No. 17 (*Streptomyces aureus*) were applied onto paper discs and analysed against No. 63 (*Streptomyces antibioticus*) and No. 49 (*Streptomyces sulphureus*), respectively. In both cases, the extracts showed distinct stimulation of antibiotic production of the receiver strains, while the extracts themselves showed no antibiotic activities (Fig. 3).

Although signalling by γ-butanolide-type autoregulators play an intraspecific role in controlling physiological and/or morphological differentiation in each *Streptomyces* species, our studies have revealed that interspecific signalling is a general feature in this group of bacteria. The frequency may be under-estimated because of the limited experimental conditions used in these studies. The high frequency among the fresh isolates implies that the interaction is more frequent in natural environments. GRÄFE *et al.*[4,5] reported previously that anthracycline production of a mutant of *S. griseus* was induced by γ-butyrolactones produced by several other *Streptomyces* species, and our previous studies showed that A-factor or A-factor-like substances were produced by several *Streptomyces* species other than *S. griseus*[6]. Thus γ-butyrolactone-type factors may serve as interspecific stimulators although in this study none of the receiver strains responded to A-factor or to other γ-butyrolactones. We suggest that different *Streptomyces* species may show symbiotic behavior by sharing a common signalling substance(s) to adjust their activities for secondary metabolism and cell differentiation. Further studies on the chemical structures of stimulatory factors will provide significant information in understanding the communication mechanism among *Streptomyces* species in the environment.

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KENJI UEDA*
SHIHO KAWAI
HIRO-OMI OGAWA
AZUSA KIYAMA
TERUYOSHI KUBOTA
HIROKO KAWANOBE
TERUHIKO BEPPU

Department of Applied Biological Sciences, Nihon University
Kameino 1866, Fujisawa 252-8510 Japan

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