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Novel Function of Cytokinin: A Signaling Molecule for Promotion of Antibiotic Production in Streptomycetes

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Abstract Cytokinin has been known to act as a plant hormone to promote cell division and function in diverse processes in plant growth and development. Besides being produced in plants, it is also produced by various bacteria and fungi; however, its ecological significance is still unclear. In this report, we present an interesting finding that *trans*-zeatin riboside (tZR), a naturally occurring cytokinin compound, increased antibiotic production in many different streptomycetes, including *Streptomyces coelicolor* M130, *S. pristinaespiralis* ATCC 25486, *S. violaceoruber* Tu22, *S. antibioticus* ATCC 11891, and *S. griseus* IFO 13350. *In vitro* plate assays showed that the addition of 100 μM tZR increased the growth inhibition of *Pseudomonas syringae pv. syringae*, a plant pathogen, by *S. griseus*, a streptomycin producer. We suggest that cytokinin could act as a signaling molecule for antibiotic production in streptomycetes, a group of rhizosphere bacteria.

Key words: Antibiotics, cytokinin, streptomycetes, tZR

Gram-positive soil bacteria of the genus *Streptomyces* is a rich source of antibiotics [2, 7, 9, 13, 14], which are one of the most important secondary metabolites used in clinical, veterinary, and agricultural fields. Regulation of antibiotic biosynthesis has been known to be exerted at different levels and, among them, small signaling molecules have been well known to switch on antibiotic production. For example, A-factor, which is produced in *S. griseus*, is a diffusible molecule that triggers streptomycin production at a critical concentration [10]. Most recently, a structurally new autoinducer, the PI factor, has also been reported, which can elicit pimaricin production in *Streptomyces natalensis* [12], and cAMP and ppGpp were also reported to be signaling molecules that can trigger antibiotic production in streptomycetes [4, 15].

During our studies on the regulation process of antibiotic biosynthesis in streptomycetes, we found that S-adenosylmethionine (AdoMet) stimulates antibiotic production in various streptomycetes [5], and we suggested that SAM may play a role as a signaling molecule in eliciting the secondary metabolism in streptomycetes. We have been interested to study the role of the adenylate pool in the regulation of cellular metabolism in streptomycetes and have carried out further studies by examining the effect of various adenine derivatives on the antibiotic production of various streptomycetes. Interestingly, we found that tZR, a compound belonging to the cytokinin family that is well known as a plant hormone, can stimulate antibiotic production in diverse streptomycetes, suggesting a novel function of cytokinin as a signaling molecule in the regulation of secondary metabolism in streptomycetes.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains used in this study are listed in Table 1. Five antibiotic producers were examined for actinorhodin, pristinamycin, granaticin, oleandomycin, and streptomycin production [6, 10, 16–18].

Assay of Actinorhodin

To prepare vegetative inoculation, the cells from a TSB agar plate were added to 50 ml of R2YE medium in a 250-ml flask. The seed culture was obtained by culturing for 48 h at 28°C with 240 rpm, after which 30 μl of broth from this culture was streaked on TSB agar plates and incubated at 28°C for 7 days. Alternatively, main culture

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Table 1. Bacterial strains used in this study.

Strains	Characteristics	References
***Streptomyces* spp.**		
S. coelicolor M130	Actinorhodin producer	[6]
S. pristinaespiralis ATCC25486	Pristinamycin producer	[15]
S. violaceoruber Tu22	Granaticin producer	[5, 16]
S. antibioticus ATCC11891	Oleandomycin producer	[17]
S. griseus IFO 13350	Streptomycin producer	[8]
Test strains		
Bacillus subtilis ATCC6633	Test strain for granaticin	In this study
Micrococcus luteus ATCC10240	Test strain for oleandomycin	[17]
Staphylococcus aureus	Test strain for pristinamycin	[15]
Plant pathogen		
Pseudomonas syringae pv. syringae	Inhibition of growing and desiccation of different parts in successive stages in plants.	In this study

was proceeded with 1% inoculation size of seed culture. The amount of actinorhodin was determined by the method described by Kieser *et al.* [8].

Exogenous Treatment of *trans*-Zeatin Riboside (tZR), Isopentyladenosine (iPA), *trans*-Zeatin (tZ), Adenosine, Adenine, and Ribose

All the above compounds were purchased from Sigma, and they were added individually at various final concentrations 12 h after inoculation (see individual figure legends). Strains were cultured at 28°C for 5 days, and samples were taken for determination of actinorhodin production and antibacterial activities.

Assay of Antibacterial Activity

Antibacterial activities were determined by the agar disc diffusion assay method. The culture broth was extracted with a solvent and applied to 8-mm paper discs on 1% nutrient agar (Sigma) spread with an overnight culture of a corresponding indicator. The diameters of the inhibition zones in the indicator bacteria lawn were assessed in triplicate experiments. Assay of antibacterial activity of pristinamycin, granaticin, oleandomycin, and streptomycin was done as previously described [6, 10, 16–18].

In Vitro Plate Assay of tZR by Growth Inhibition of *Pseudomonas syringae pv. syringae*

S. griseus IFO13350 was used in this assay system as an antibiotic producer. *S. griseus* was cultured in YMPD liquid medium at 28°C for 2 days, and 75 µl of the culture broth was loaded on a 8-mm paper disc and the paper disc was placed on agar plates with or without 100 µM final concentration of tZR. The plates were incubated at 28°C for 2 days, and then overlayed with 1% *P. syringae pv. syringae* in 10 ml of nutrient agar. Inhibition zones were observed after 2 days of further incubation of the plates at 28°C.

RESULTS

Cytokinin Promotes Antibiotic Production in Streptomycetes

Effect of tZR on actinorhodin production in *S. coelicolor* M130, which is a model system in the studies of antibiotic biosynthesis in the *Streptomyces* genus, was investigated. As shown in Fig. 1, tZR at various concentrations (2 µM, 50 µM, and 100 µM) was examined, and it was found that actinorhodin production was increased by tZR at a concentration as low as 2 µM. The highest actinorhodin production (3-fold compared with control) was detected when treated with tZR. Treatment of tZR at 100 µM concentration was less effective than 50 µM, implying that tZR acts as a signaling molecule and its optimal concentration appears to be 50 µM, at least under the condition employed.

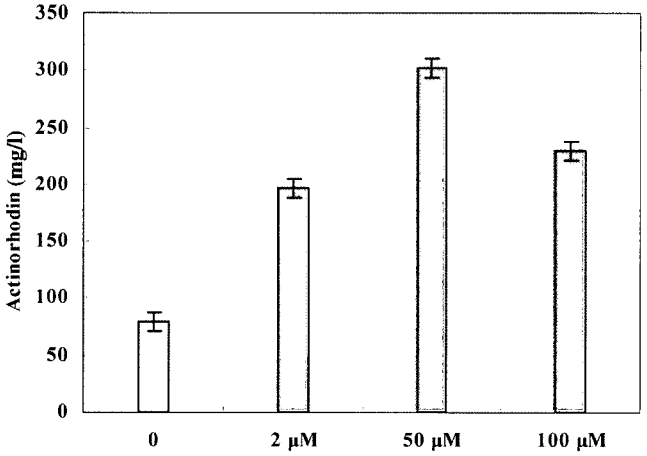

Fig. 1. Actinorhodin production in R2YE liquid medium of *S. coelicolor* M130.
tZR at different final concentrations was added into the culture medium of *S. coelicolor* M130 12 h after inoculation. Cells were grown at 28°C for 5 days.

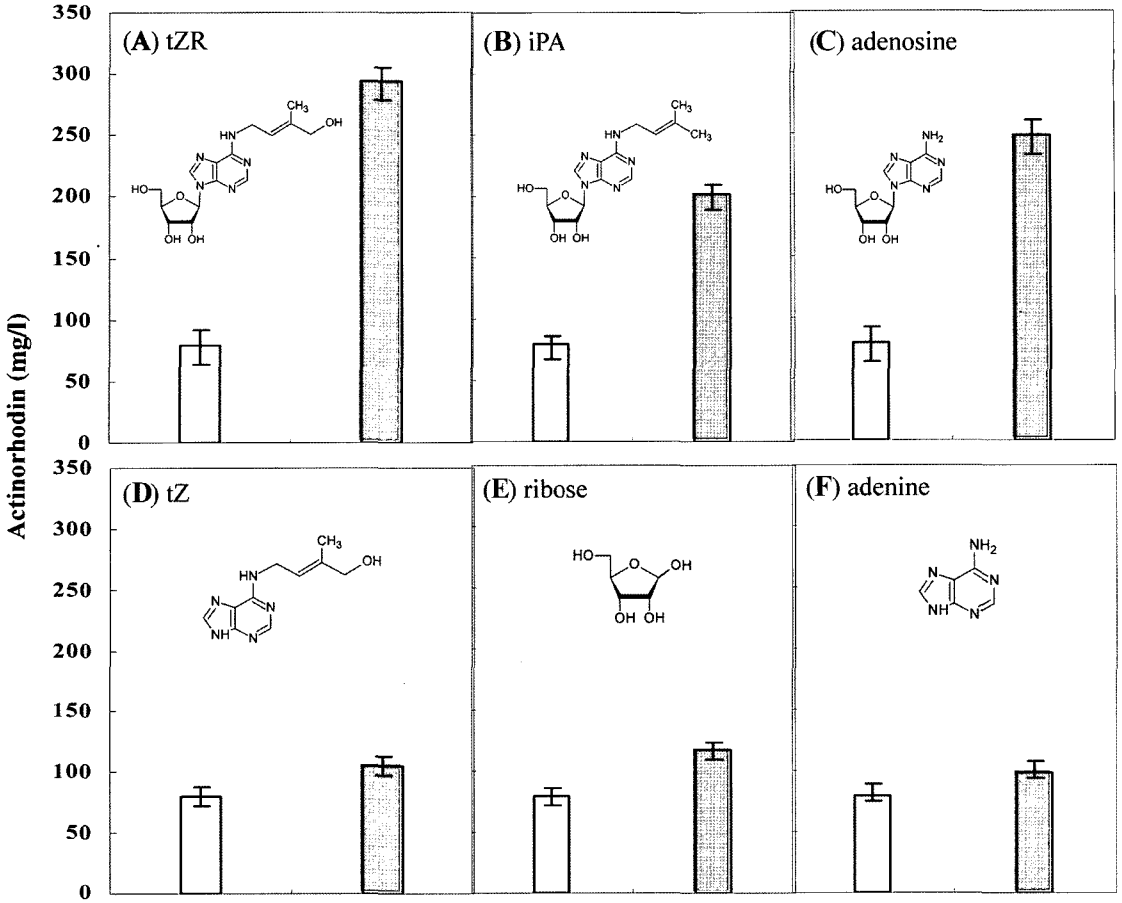

Fig. 2. Effects of various compounds to enhance actinorhodin production in *S. coelicolor* M130.
Actinorhodin production was investigated after treatment with exogenous (**A**) tZR, (**B**) iPA, (**C**) adenosine, (**D**) tZ, (**E**) ribose, and (**F**) adenine in *S. coelicolor* M130. Unfilled columns represent control samples and filled columns represent samples treated with 50 μM each materials. Cells were grown in a TSB liquid medium and treated with each exogenous compound, as described in Materials and Methods. All plots represent an average of at least three independent experiments. Error bars represent standard deviations.

There are many different kinds of compounds in plants that have cytokinin activities, such as tZR, isopentyladenosine (iPA), and *trans*-zeatin (tZ), *etc.* Addition of 50 μM iPA resulted in a 2.5-fold increase in actinorhodin production. Structurally, iPA and tZR differ only in the moiety that links to the adenosine moiety, and their structures contain an adenosine moiety. To reveal the structure-activity relationship, tZ and ribose were also examined for their abilities to activate antibiotic production; however, no enhancing effect was found. These results showed that the linkage of the tZ and ribose is very important for this activity. We then examined the effect of adenosine on actinorhodin production, and found that addition of adenosine could result in a 2.5-fold increase of actinorhodin production; however, no increasing effect was observed when adenine was examined (Fig. 2).

Widespread Promotion of Antibacterial Activities by tZR in Streptomycetes

To examine whether the activation of antibiotic production by tZR is limited in actinorhodin or rather whether it constitutes a widespread phenomenon in the communication of soil microorganisms, four additional antibiotic producers, including three antibiotics such as oleandomycin, pristinamycin and granaticin producers, as well as one aminoglycoside antibiotic, a streptomycin producer, were selected to investigate the effects of tZR on their antibacterial activities. Antibacterial activities of these four streptomycetes were increased 2~3-fold by tZR, but in different patterns (Fig. 3): The optimum concentration was 100 μM, 50 μM, 100 μM, and 100 μM for antibacterial activities of oleandomycin, pristinamycin, granaticin, and streptomycin producer, respectively, while showing no significant difference between the four *Streptomyces* strains. Granaticin and streptomycin producers at concentrations less than 50 μM showed very little response to tZR, whereas 50 μM tZR presented the highest antibacterial activity in the pristinamycin producer. The oleandomycin producer, however, demonstrated a 1.5-fold increase, even at 2 μM tZR.

The same stimulatory effect was also found with the treatment of iPA and adenosine. However, no effect was

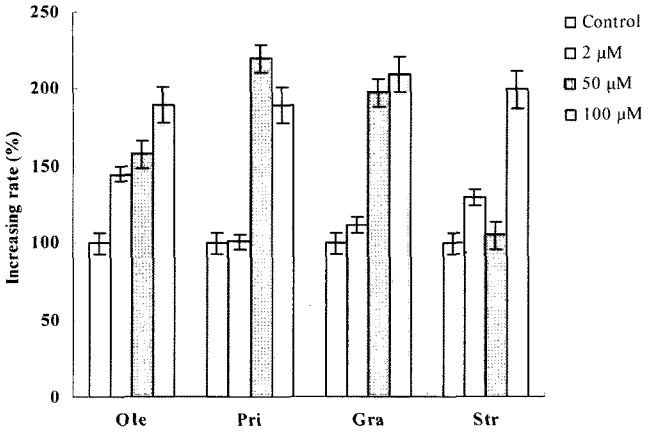

Fig. 3. tZR increased antibacterial activities in various streptomycetes.

Antibiotic-producing strains, including *S. antibioticus*, *S. pristinaespiralis*, *S. violaceoruber* Tu22, and *S. griseus*, oleandomycin producer (Ole), pristinamycin producer (Pri), granaticin producer (Gra), and streptomycin producer (Str) were examined after addition of tZR. The production level of a non-addition control was set as 100%. The optimum concentration of tZR was 100 μM for oleandomycin, granaticin, and streptomycin production, and 50 μM for pristinamycin production.

found, when ribose, tZ, or adenine was added to the antibiotic production medium (data not shown).

Growth Inhibition of *P. syringae pv. syringae* was Enhanced by tZR *In Vitro*

To further test the possible effect of tZR on antibiotic production and pathogen resistance, we set up a model in which *S. griseus* was used as an antibiotic-producing strain, and *P. syringae pv. syringae* was used as an indicator of pathogen. After growing *S. griseus* in tZR-containing plates for 2 days, we found that the inhibition zone of *P. syringae pv. syringae* was apparently much larger than that

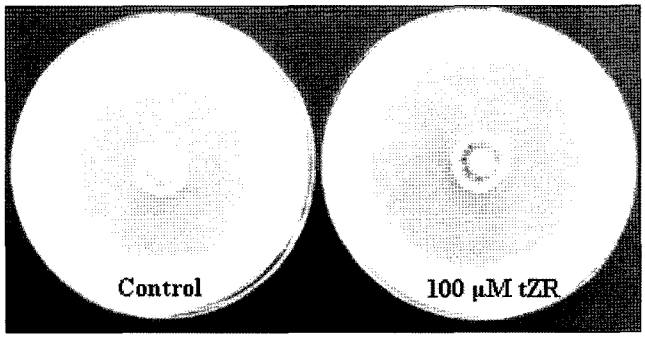

Fig. 4. *In vitro* plate assay of tZR in the growth inhibition of *Pseudomonas syringae pv. syringae*. *S. griseus* IFO13350 was used as an antibiotic producer and was loaded on a paper disc.

The plates, containing YMPD medium with or without tZR, were seeded with *Pseudomonas syringae pv. syringae*. See Materials and Methods for more detail. Plate cultures were photographed after incubation at 28°C for 2 days.

of the control (Fig. 4). However, the growth of *S. griseus* was not different from the control.

DISCUSSION

In the present study, we observed an interesting finding that cytokinin, in addition to its well-known function as a plant hormone, can elicit antibiotic production in streptomycetes. It was not clear until now whether there is a special receptor in streptomycetes cells to respond to exogenous cytokinin, and how cytokinin is metabolized in streptomycetes. It has been known that about 200 species of microorganisms, referred to as PGPR (Plant-Growth Promoting Rhizobacteria), can also produce cytokinin [1, 11]. However, the ecological significance of the secretion of cytokinin by microorganisms is not clear. We suggest that the excretion and allocation of this molecule produced by PGPR into a soil environment might transfer an interdisciplinary signal among a complex community, triggering different biochemical machineries.

In a previous study, we found similar results in which intracellular SAM increased antibiotic production in streptomycetes. Structurally, SAM resembles tZR in that it also has an adenosine moiety. Studies of different compounds containing the adenosine moiety revealed that adenosine, tZR, and iPA increased actinorhodin production, whereas adenine, tZ, and ribose did not show increasing effects. Therefore, the adenosine moiety appears to be important factor in the enhancing activity of tZR in actinorhodin production. We found that tZR was more effective than tZ, and it might be due to either higher solubility of tZR than tZ, as documented in plant system [3], or the fact that the ribose moiety is essential as a signaling molecule. Further experiment seems to be necessary to answer the question of why tZR is more effective than iPA and adenosine. tZR was also demonstrated to show increasing effects on the production of a variety of antibiotics. Collectively, these results imply that the adenosine moiety of tZR, which also exists in SAM and SAH, is indispensable for the activation of antibiotic production.

The effect of tZR on the growth inhibition of *P. syringae pv. syringae* by *S. griseus* on agar plate suggested that tZR as a signaling molecule increased antibiotic production in streptomycetes and then further strengthened the growth inhibitory effect on *P. syringae pv. syringae* by *S. griseus*. Although the real ecological system should be much more complicated, it is quite possible that such cell-cell communications through tZR may also occur in a natural soil environment. Further studies will unveil more detail of the involvement of cytokinin in interspecies communication among soil microorganisms.

In conclusion, our data provide a direct demonstration that tZR, although not reported to occur naturally in cell metabolism of streptomycetes, can act as a signaling molecule for antibiotic production in a variety of streptomycetes.

Acknowledgments

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