Stimulatory Effect of Copper on Antibiotic Production and Morphological Differentiation in *Streptomyces tanashiensis*

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The Gram-positive bacterial genus *Streptomyces* has two characteristic features; one is its productivity of a wide variety of secondary metabolites such as antibiotics or other biologically active compounds, and the other is its ability to perform cellular-differentiation from substrate hyphae into aerial mycelium and spores[1,2]. These phenotypic features of *Streptomyces* are known to be induced by extracellular signal molecules produced by the organism itself. For example, A-factor, a γ-butyrolactone derivative, is produced by *Streptomyces griseus* and induces both its streptomycin production and initiation of development into aerial mycelium at a concentration as low as 10^{-9} M[3~5]. Involvement of a series of A-factor analogues[6] as well as some polypeptidic signals[7] have been observed with secondary metabolism and morphogenesis in various *Streptomyces* spp. During the course of our systematic screening targeting such extracellular signalling substances in *Streptomyces* spp., we found that low concentrations of copper ion plays a crutial role in antibiotic production and sporulation in several strains. Here we describe typical phenotypic features of a copper-responding mutant strain derived from *Streptomyces tanashiensis*.

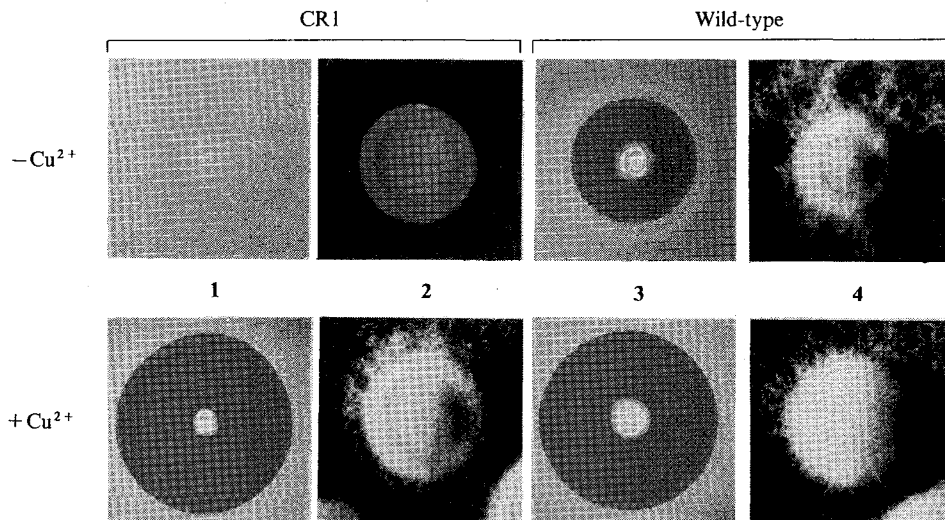
Summary of the strategy by which we obtained the mutant and revealed its copper-responding phenotype is as follows: the parental strain *S. tanashiensis* st-25-4 was obtained from the culture collection of the Laboratory of Fermentation and Microbiology, the University of Tokyo. Spores of the strain were mutagenized by UV-irradiation to reduce surviving cells to 0.1% and plated on BENNETT's-glucose agar plates containing 1 g yeast extract (Difco Laboratories), 1 g meat extract (Kyokuto), 2 g NZ-amine (Difco Laboratories) and 10 g glucose in 1 liter (pH 7.2). We isolated spore-deficient colonies and spread them on fresh BENNETT's-glucose agar plates to form an indicator lawn, onto which we placed agar discs cut out from a fully-grown plate culture of the parental strain. Most of the bald mutants showed a distinctly sporulating zone around the discs which were supposed to contain metabolites of the wild-type cells. We observed a similar effect with standard paper discs containing the culture filtrate of the wild-type strain. However, we soon noticed that similar recovery of sporulation was induced by agar discs without any growth of the strain st-25-4 or by paper discs without any culture filtrate. Therefore, we tested substances possibly contained in those materials, and finally found that low concentrations of Cu^{2+} ion fully replaced both the agar discs and paper discs to recover sporulation in these mutants. We speculated that the reason why the agar discs or paper discs themselves gave a positive response on the mutants was that the metalic cork-borers made of copper alloy used for disc preparation caused contamination with low but sufficient concentrations of Cu^{2+}. As we expected, agar discs prepared by glass-made tubes did not show sporulation-inducing activity.

The copper-dependent bald mutants were derived at a high frequency of 2×10^{-2} by UV irradiation from *S. tanashiensis* st-25-4. We further investigated characteristics of one of these mutants, *S. tanashiensis* CR1. Fig. 1 shows the phenotypic feature of the CR1 and its parental strain (wild-type) on BENNETT's-glucose agar. CR1 was defective in not only sporulation but also antibiotic production as detected by the growth inhibitory effect against *Bacillus subtilis* (panel 1 and 2, upper). On the other hand, both deficiencies were recovered by supplementing 1 μM of $CuSO_4$ to the medium (panel 1 and 2, lower). In the wild-type strain, the addtion of copper also caused an elevation of antibiotic production (panel 3, lower) and acceleration of sporulation. The wild-type colonies grown on BENNETT's-glucose agar supplemented with 1 μM $CuSO_4$ started to form spores 6 hours earlier than those without supplementation (data not shown). These results indicate that the stimulation by copper is effective not only on the CR1 mutant but also on the wild-type strain.

We also tested $CuCl_2$, CuCl, copper (II) acetate, copper (II) gluconate and copper powder, all of which showed the same stimulatory effect as $CuSO_4$. On the other hand, other metallic compounds [$FeSO_4$, $MnSO_4$, $ZnSO_4$, $NaMoO_4$, $CaCl_2$, $[Co(NH_4)_6]Cl_3$, $Al_2(SO_4)_3$, $BaSO_4$, $HgCl_2$, $NiSO_4$, $AgSO_4$, LiCl, RbCl, $CdCl_2$, $P_2O_5 24WO_3$, NH_4VO_3, GeO_2, Ti powder, $BeSO_4$, $SrCl_2$, K_2PtCl_6 and $Na(AuCl_4)$] did not show activity. These results indicate that copper is the unique factor that causes the stimulatory effects on both antibiotic production and sporulation in *S. tanashiensis*.

When *S. tanashiensis* CR1 was cultured aerobically in

Fig. 1. Antibiotic production and sporulation of *S. tanashiensis* CR1 and its parental strain.

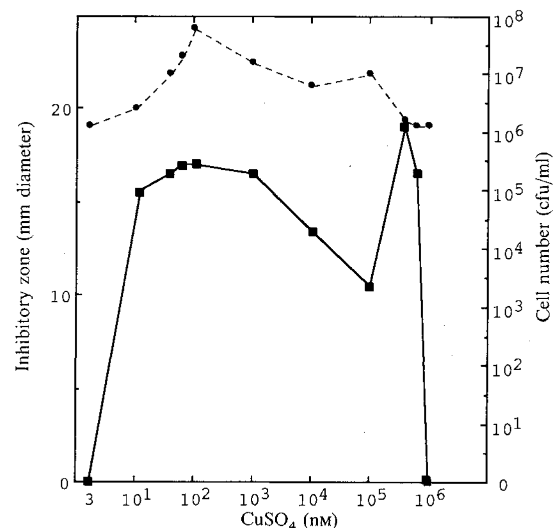


S. tanashiensis CR1 (panel 1 and 2) and the wild-type strain (panel 3 and 4) were grown on BENNETT's-glucose agar with (lower) or without (upper) 1 μM $CuSO_4$ at 28°C for 3 days, and each antibiotic productivity (panel 1 and 3) and colony surface (panel 2 and 4) were photographed. *B. subtilis* was used as an indicator for antibiotic activity. Colony surface was observed by a stereomicroscope (Olympus SZH10).

liquid BENNETT's-glucose medium, no sporulation occured but antibiotic production was observed. As shown in Fig. 2, the exogenous $CuSO_4$ markedly enhanced antibiotic production without affecting cellular growth using concentrations between 10 nM and 700 μM. The endogenous concentration of Cu^{2+} in BENNETT's-glucose medium was found to be approximately 3 nM by atomic absorption analysis (Plasma Spectrometer SPS1200VR, SEIKO Instruments Inc.). Therefore we conclude that the minimum concentration of Cu^{2+} for the stimulatory effect is between 3 to 13 nM. Inhibition of cellular growth was observed with 1 mM and higher concentrations of $CuSO_4$. The antibiotic measured in this experiment was extracted with ethyl acetate. Although *S. tanashiensis* type strain is known to produce luteomycin[8], we do not have reliable information on the chemical structure of the antibiotic produced by this strain.

We speculate that the CR1 strain is a mutant lacking the ability to transport and accumulate copper into the cell, which might be reqired for a regulatory component to induce both antibiotic production and sporulation. KIESER *et al.*[9] reported that addition of 2 μM of Cu^{2+} to media improved sporulation of *Streptomyces lividans* 66. We also observed with several strains including *S. griseus* and *S. coelicolor*, that acceleration and elevation of both secondary metabolite formation and sporulation were caused by the addition of Cu^{2+} into the media. We speculate that Cu^{2+} specifically plays a crucial role for

Fig. 2. Antibiotic production and cellular growth of *S. tanashiensis* CR1 with various concentrations of $CuSO_4$.



Antibiotic was extracted with ethylacetate from 1 ml of each culture filtrate. After evaporation, each sample was dissolved in 50 μl of sterile water and applied onto paper discs to measure the anti-microbial activity against *B. subtilis*. The activities are represented by the diameter of inhibitory zones (solid line). Cellular growth was determined by measuring colony forming units of each culture (dashed line).

secondary metabolism and morphogenesis in most *Streptomyces* species. A stimulatory effcect of copper has been described for morphogenesis of fungi[10~12], in

which copper is assumed to exert its effect through the activation of phenol oxidases. Further analysis will elucidate whether a similar mechanism is widely distributed among *Streptomyces*.

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