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NOTE

Formation and Dispersion of Mycelial Pellets of *Streptomyces coelicolor* A3(2)

Yul-Min Kim and Jae-heon Kim*

Department of Microbiology, Dankook University, Anseo-Dong San 29, Cheonan 330-714, Korea

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The pellets from a culture of *Streptomyces coelicolor* A3(2) that were submerged shaken were disintegrated into numerous hyphal fragments by DNase treatment. The pellets were increasingly dispersed by hyaluronidase treatment, and mycelial fragments were easily detached from the pellets. The submerged mycelium grew by forming complexes with calcium phosphate precipitates or kaolin, a soil particle. Therefore, the pellet formation of *Streptomyces coelicolor* A3(2) can be considered a biofilm formation, including the participation of adhesive extracellular polymers and the insoluble substrates.

***Key words*:** biofilm, dispersiveness, DNase, hyaluronidase, pellet, *S. coelicolor* A3(2)

The genus *Streptomyces* displays a colony morphology, which is an exciting model system for prokaryotic differentiation (Kalakoutskii and Agre, 1976). Therefore, extensive studies have been carried out on various aspects of colony differentiation of this bacterium. *S. coelicolor* A3(2) is the most effectively studied streptomycete. Its genetic program leading to sporulation of aerial mycelium has become well known, and possibly in other species an analogous system might also be functional (Chater, 1998).

In the submerged culture, however, the colony differentiation does not occur as it does for surface cultures. Instead, many strains grow to form the mycelial clumps with varying degrees of complexity, e. g., dispersed mycelium, mycelial aggregation, or ball-shaped pellet of dense network of mycelium (Cox *et al.*, 1998). The pellet formation has been thought not as a part of the differentiation process, but as a result of entanglement and subsequent interweaving of numerous branching hyphae (Whitaker, 1992). This pellet growth is not suitable for physiological study, because there are heterogeneous physiological states even within a pellet; the growth occurs only at the outer surface while the growth ceases at the central region due to nutrient limitation or even autolysis of the mycelium (Braun and Vecht-Lifshitz, 1991).

Therefore, studies that avoid pellet formation and achieve the dispersion of growth with free mycelium have been undertaken, because this is preferred for the lucid illustration of physiological responses of the bacterium (Hopwood *et al.*, 1985; Hobbs *et al.*, 1989). In trying to achieve dispersed growth, we found out that cultivation at 37°C allowed increased dispersiveness in the growth of *S. griseus* (Kim and Hancock, 2000). In the present paper, we describe the role of some solid matters and adhesives in the formation of a *Streptomyces* pellet.

S. coelicolor A3(2) M145, a wild type strain, was used throughout this work. The spore solution was prepared from a yeast-extract malt-extract agar plate culture (YEME; yeast extract 4 g, malt extract 10 g, agar 15 g, deionized water 1L, pH 7.2), and stored at -20°C, until it was used as an inoculum (Hopwood *et al.*, 1985). Spores were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of YEME liquid medium to give the final spore concentration of about 10^4 spores/ml, and then the final mixture was incubated at 200 rpm, 30°C. The compact spherical pellets (usually 2 mm in diameter) appeared after 2 days, and the culture fluid remained transparent. Although it is believed that the pellet formation begins with clumping and subsequent entangling of hyphae, we needed more explanations to understand how growing pellets are prevented from being crumbled and how hyphae are stuck together. There should be substances that are responsible for the relatively strong cohesion of hyphae. A recent paper dealing with biofilm formation of *Pseudomonas aeruginosa* describes extracellular DNA as a major component of the matrix that holds bacteria together. DNase I treatment dissolved the existing biofilm and at the same time, inhibited a new biofilm formation (Whitchurch *et al.*, 2002). If we regard a pellet as a biofilm, then it might be possible that DNA will carry out the same role in the pellet of *Streptomyces.* In order to test

* To whom correspondence should be addressed.
(Tel) 82-41-550-3452; (Fax) 82-41-551-9229
(E-mail) jaehkim1@dankook.ac.kr

this possibility, pellets were harvested by gravitation and were washed twice with deionized water. About 10 washed compact pellets were resuspended into 100 µl of reaction buffer (40 mM Tris-HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$, pH 8.0) and were incubated with 10 units of RQ1 RNase-free DNase (Promega, USA) for 4 h at 37°C. After this treatment, the compact pellets were disintegrated into numerous fragments of hyphae (Fig. 1). Two restriction endonucleases, *Eco*R1 and *Bam*H1 (Promega, USA), were also examined for comparison, but they showed little dispersing effects.

We further examined whether DNA was eluted from pellets into the enzyme reaction mixtures. As shown in Fig. 2, relatively short smeared DNA bands, which were smaller than 2 kb, were detected as major bands because they were detected together with the weak chromosomal bands, if ever, in the reaction buffers (Promega, USA). Apparently, these major DNA bands were not digested by the restriction enzymes (lanes 1 and 3), while being digested by DNase (lane 5).

Therefore, it appeared that there were many small DNA fragments in the compact pellets, and their breakdown was linked to the dispersion of the pellets. On the origin of these DNA fragments, we supposed that they were produced during autolysis of the inner part of the pellet and remained as a component of the lysate, as the internal hydrolysis of pellets was confirmed for streptomycetes (Braun and Vecht-Lifshitz, 1991).

All buffers used for enzyme reactions included Tris, and pHs of these buffers were slightly alkaline, a condition that is required for the hydrolysis of *Streptomyces* (Kim and Hancock, 2000). Therefore, although we did not examine morphology, the lysis of the mycelium should have occurred in this experiment, so that some chromosomal DNA would appear. However, we were able to immediately examine the effect of DNase buffer on the growth of *Streptomyces*. For a clearer evaluation of the buffer effect, we used a liquid minimal medium (MM) containing 0.5 g of L-asparagine, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 10 g of glucose and 1 L of deionized water (Hopwood *et al.*, 1985). As soon as the MM was mixed with DNase buffer, the medium became turbid owing to the precipitation of calcium phosphate. In this modified MM, *S. coelicolor* A3(2) also grew in the form of pellets. Interestingly, most of the pellets were in the complex form of the mycelium and calcium phosphate precipitate (Fig. 3). When these pellets were treated with 1 N HCl or 0.5 M EGTA, a calcium chelating agent, only calcium phosphate disappeared while the remaining mycelial network became more clearly visible from the complex (Fig. 4). Our results indicate that the insoluble form of calcium salts functions as

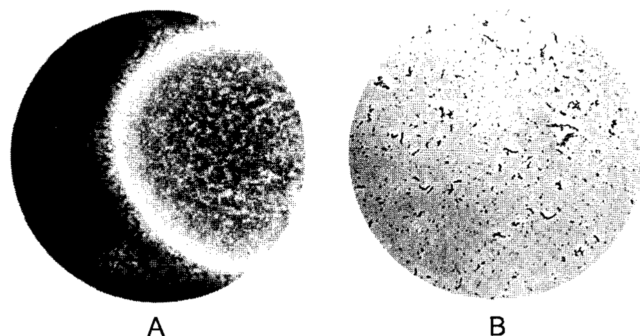

Fig. 1. Microscopic observation of a pellet of *S. coelicolor* A3(2). A compact pellet before DNase treatment (A) and numerous fragments of hyphae after DNase treatment (B).

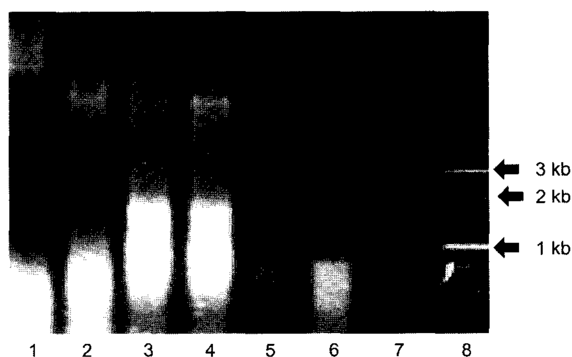

Fig. 2. Agarose gel electrophoresis of DNA eluted from *S. coelicolor* A3(2) mycelial pellets. Lanes 1, +*Bam*HI; 2, +*Bam*HI buffer; 3, +*Eco*RI; 4, +*Eco*RI buffer; 5, +DNase; 6, +DNase buffer; 7, Culture filtrate; 8, 1 kb ladder(Promega.).

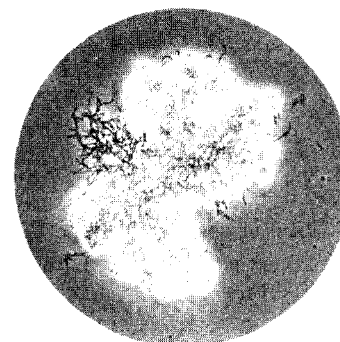

Fig. 3. Photomicrograph of a pellet (mycelium-calcium phosphate complex) of *S. coelicolor* A3(2) grown in a modified minimal medium.

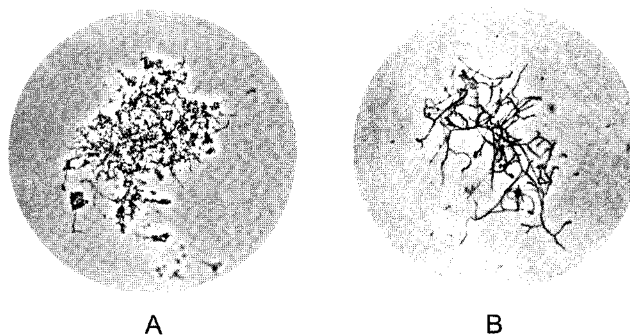

Fig. 4. Visualization of the mycelial network in a mycelium-calcium phosphate complex of *S. coelicolor* A3(2) by HCl (A) and EGTA (B).

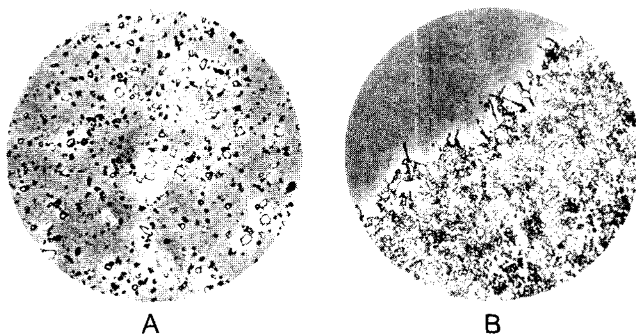

Fig. 5. The growth of *S. coelicolor* A3(2) in the presence of kaolin particles. Free kaolin particles before growth(A) and mycelium-kaolin complex(B).

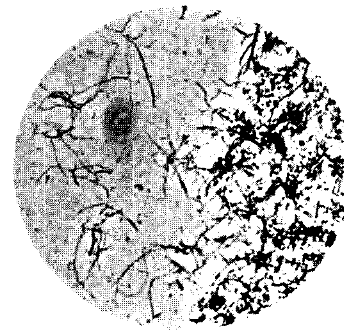

Fig. 6. Fragmentation of a pellet of *S. coelicolor* A3(2) by hyaluronidase.

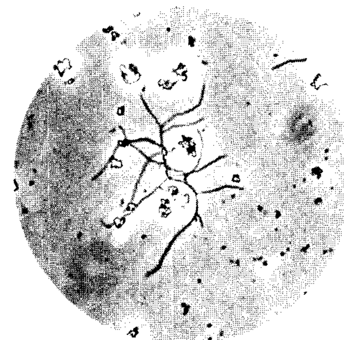

Fig. 7. Dissociation of a mycelium-calcium phosphate complex by hyaluronidase.

a physical support in the formation of pellets. The importance of the calcium ion has been previously reported in terms of hyphal clumping of streptomycetes (Braun and Vecht-Lifshitz, 1991; Miguelez *et al.*, 1993). Thereupon, the effect of kaolin, which is the principal particle of clay, was examined. Kaolin particles are similar to or much smaller than the bacterial cells in size (< 2 μm). The aggregation of clay particles with microorganisms are known to be a determinant factor of soil structure. As shown in Fig. 5, when kaolin (Sigma, USA) was added into YEME or modified MM, particles were uniformly distributed into the liquid culture medium before growth, but after the growth, nearly all kaolin particles were found in the form of the mycelium-kaolin complex.

Streptomycetes are soil microbes, and it has been known that soil particles are attached to the microbes leading to the formation of the soil-microbe aggregation. In nature, they most likely do not grow by developing the swimming pellets as shown in liquid cultures of the lab. Rather, they form sessile biofilm on the surfaces of large soil grains such as sands (Lynch, 1983). Our result that calcium precipitate and kaolin were used as solid substrates is in accordance with the natural life form of streptomycetes.

It was reported that a colony of *Streptomyces* was surrounded by a membrane-like surface structure, and the inter-hyphal space in a colony was filled with unknown extracellular substances (Garcia, 1995). Similar structures have been found in the biofilm of other bacteria (Wai *et al.*, 1998). Polysaccharides would be a component of the extracellular substances, and one example is hyaluronic acid of *Streptococcus pyogens,* which provide adhesive forces to form aggregation of cells. This functions as the protective shield that allows streptococcal cells to be more tolerant against toxic oxygen metabolites (Cleary and Larkin, 1979).

When the pellets of *S. coelicolor* A3(2) were treated with hyaluronidase from bovine testes(Sigma, USA), the pellets became very fragile, and numerous mycelial fragments fell out of the treated pellet (Fig. 6). When the calcium phosphate-mycelium complex was also treated with the hyaluronidase, two components of the pellet were disjoined, and a free network of mycelium was disclosed (Fig. 7). These results present the possibilities that hyaluronic acid is extracellularly produced by *S. coelicolor* A3(2), and this acid has a role as adhesive in the formation of pellets.

As we mentioned above, the pellet formation should require various kinds of attractive forces to hold the entire mycelial network together in a tight manner. This is the first report that revealed that extracellular substances, such as DNA and hyaluronic acid, as well as solid matrixes, such as calcium precipitate, were cooperatively involved in the formation of pellets of the submerged culture of *S. coelicolor* A3(2).

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