Joural of Microbiology and Biotechnology
Vol. 5, No. 1, 22-25, 1995

Optimal conditions for pigmentation in *Bacillus licheniformis* SSA3 and cloning of a DNA fragment involved in pigment production

KIM, JONG-KYU*, OK-SUN SHIN AND SANG-JUN LEE

Department of Applied Microbiology, Yeungnam University, Kyongsan 712-749, Korea

Bacillus licheniformis SSA3 can produce a dark-brown antimutagenic pigment. The optimal conditions for production of this pigment are reached at 0.1% tyrosine, in pH 6-8, within 7-9 days, at 30℃, and in aerobic condition. We cloned a DNA fragment involved in pigment synthesis from *Bacillus licheniformis* SSA3 using a mutant strain. The cloned DNA was 7kb in size, which can produce the same pigment even in *E. coli*.

We confirmed that *Bacillus licheniformis* SSA3 can synthesize a novel dark-brown antimutagenic pigment in the previous paper (8).

A similar brown pigment was isolated from *Bacillus subtilis* by Barnett *et al.* (1). They characterized the brown pigments that were formed outside the cells by a non-enzymatic pathway and produced concomitantly with sporulation of *Bacillus subtilis*.

However, it was reported that the synthesis of the dark-brown pigment by *Bacillus licheniformis* SSA3 is not linked to the sporulation by Kim and Kim (7) in this laboratory.

Therefore, we concluded that this pigment from *Bacillus licheniformis* SSA3 is different from that from *Bacillus subtilis*. We investigated the optimal conditions for this pigment production and cloned the DNA fragment involved in the pigment synthesis in this study.

MATERIALS AND METHODS

Strains, Plasmids and Plates

Bacillus licheniformis SSA3 and its unpigmentable mutant *Bacillus licheniformis* SSA3-2M1 (7), and *E. coli* strain C600 (9) was used.

Promoter probe vector pGR71 (6) was used as a shuttle vector for *Bacillus* and *E. coli*.

All strains were cultured in the specified minimal or nutrient media (5).

Optimal Conditions for Pigmentation

The optimal conditions for the maximal pigmentation by *Bacillus licheniformis* SSA3 were examined in the tyrosine supplemented minimal media. The cultivation of *Bacillus licheniformis* SSA3 was carried out with 5 ml volume in a test tube (1.5×18.5 cm) on a rotary shaker set at 150-200 rpm for 15 days and at 30℃ aerobically. The following conditions for pigmentation were investigated: the effect of tyrosine, the effect of initial pH, the time course of pigmentation, the effect of temperature, and the effect of aeration. The result of pigmentation in the liquid media was measured as the absorbance at 430 nm of the solution in milliliters according to Barnett *et al.* (2) and in solid media was evaluated by obsorbance.

DNA Techniques and Transformation

Recombinant DNA techniques were performed as described Maniatis *et al.* (9) except *Bacillus'* mini-prep DNA preparation and protoplast transformation. In *Bacillus* species the mini-prep DNA was prepared by adding lysozyme as in yeast as described by Maniatis *et al.* (9) and the protoplast transformation was carried out by the method of Chang and Cohen (3) except using nutrient agar plates for cell-membrane regeneration. Restriction endonucleases and T4 DNA ligase were used according to the manufacture's protocol (Boehringer Mannheim).

RESULTS AND DISCUSSION

Bacillus licheniformis SSA3 produced a novel pigment in the tyrosine supplemented minimal media (8). To optimize this pigment production, we investigated the culture conditions and analyzed this pigment synthesis at the genetic level. Then we cloned the DNA fragment involved in pigment synthesis in the unpigmentable

*Corresponding author
Key words : *Bacillus licheniformis* SSA3, pigmentating conditions, cloning of a DNA fragment

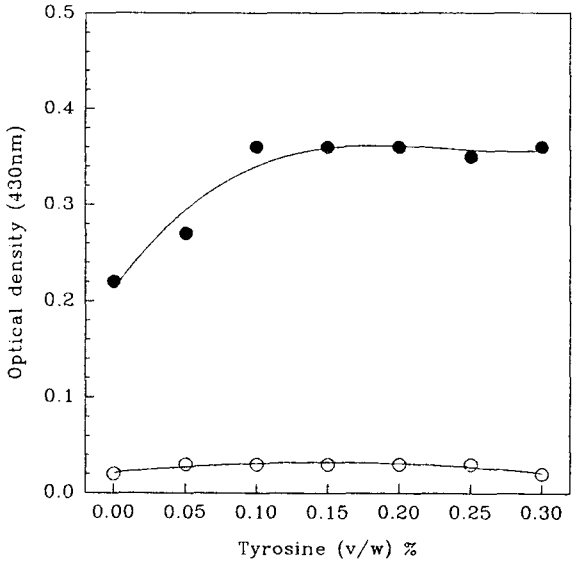

Fig. 1. Effect of tyrosine on pigment production by *Bacillus licheniformis* SSA3.
● : *Bacillus licheniformis* SSA3, ○ : Control(no inoculation)

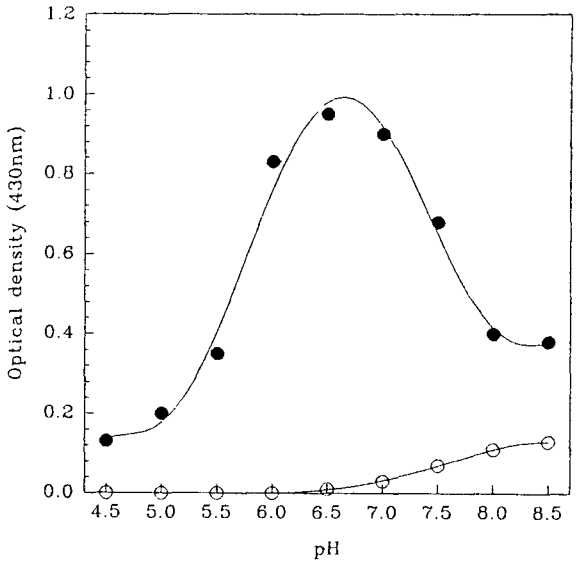

Fig. 2. Effect of pH on pigment production by *Bacillus licheniformis* SSA3.
● : Bacillus licheniformis SSA3, ○ : Control(no inoculation)

mutant.

Effect of Tyrosine

The amount of pigment produced by *Bacillus licheniformis* SSA3 in the minimal liquid media supplemented with various concentrations of tyrosine is shown in Fig. 1. The optical density (430 nm) of this pigment was increased between 0.0 and 0.1% (w/v) of tyrosine. The maximal value of tyrosine as an effective concentration was reached at 0.1% (this concentration was used in the following experiments). The maximized optical density value was not increased beyond 0.1 to 0.5% of tyrosine used, whereas the optical density of the uninoculated control was hardly changed.

The pigment production of *Bacillus licheniformis* SSA3 against the concentration of tyrosine was a first order kinetic as seen frequently in a typical enzymatic reaction. In the previous result a mutant strain *Bacillus licheniformis* SSA3-2M1 could not synthesize the dark-brown pigment in the presence of tyrosine (7). Therefore we suggest that the synthesis of this pigment is an enzymatic reaction as our previous conclusion (8).

Effect of pH

The effect of pH on pigment synthesis of *Bacillus licheniformis* SSA3 in the 0.1% tyrosine supplemented minimal liquid media was shown in Fig. 2. The pigment synthesis was dominant between neutral pH (6.0-7.5) and the highest pigment synthesis was obtained at pH 6.5.

The pH of pigmented tubes was increased up to 0.1-0.5 over the initial pH. It was reported that the increased pH is caused by the change of media utilization according to the growth of bacteria (12, 14, 31). When we consider the increased pH, the adjusted final pH of the pigmented tube reaches the range of pH 6.5-8.0. Therefore we concluded that the optimum pH for this pigment synthesis is within the range of neutral pH.

Time Course of Pigmentation

The time course of pigment synthesis by *Bacillus licheniformis* SSA3 in the tyrosine supplemented minimal liquid medium was 7 to 9 days. The optical density was abruptly increased between 5 and 7 days as shown in Fig. 3. After pigmentation, the colors of supernatant and bacteria were dark-brown.

The bacteria were isolated from the pigmented tube by centrifugation, and washed in distilled water several times by centrifugation to remove all pigment. The bacteria was also disrupted by sonication according to Christian (4). The resultant supernatant was dark-brown (data not shown). From this experiment, it was highly probable that the pigment was synthesized in the bacteria. The pigment was secreted out of the bacteria during the long culture period as reported by Schaeffer (10) rather than the pigment being synthesized outside bacteria.

According to the abrupt pigment synthesis after 5 days, we consider that this pigment was synthesized at the death phase of fully grown bacteria by the secondary metabolite related enzyme(s) rather than by the primary metabolite related enzyme(s).

Effect of Oxygen

Tests on the effect of oxygen on pigment synthesis. Several volumes of *Bacillus licheniformis* SSA3 inoculated cultures (5, 10, 15, 20 and 25 ml of tyrosine supplemented minimal liquid media) in a test tube (1.5×

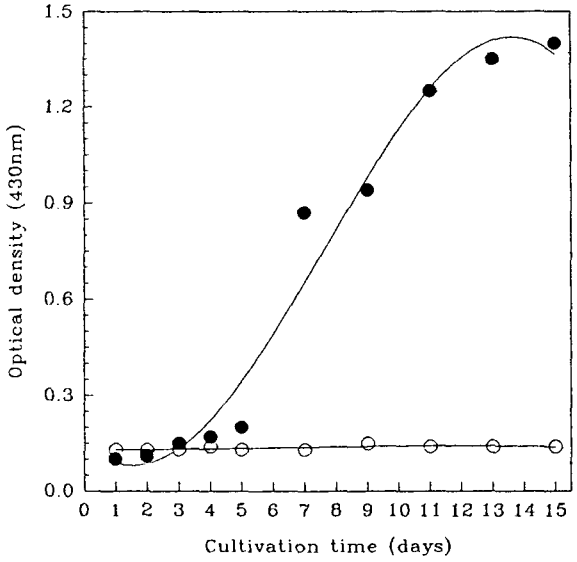

Fig. 3. Time course of pigment production by *Bacillus licheniformis* SSA3.
●: Bacillus licheniformis SSA3, ○: Control(no inoculation)

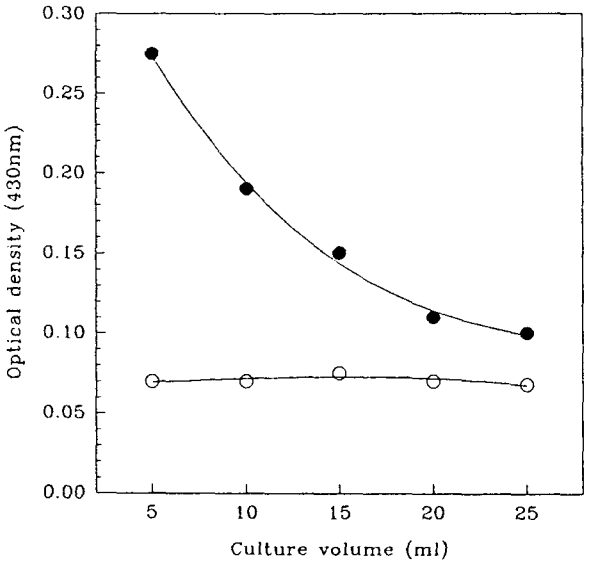

Fig. 4. Effect of oxygen on pigment production by *Bacillus licheniformis* SSA3.
●: Bacillus licheniformis SSA3, ○: Control(no inoculation)

18.5 cm) were closed with caps, and cultured by standing for 15 days without shaking at 30℃. The result showed that the 5 ml volume of culture has the highest optical density, whereas the 25 ml volume of culture has the lowest optical density (Fig. 4). It is highly probable that the synthesis of this pigment was affected by the supplied oxygen in the medium.

Effect of temperature

The effect of temperature on pigment synthesis of *Bacillus licheniformis* SSA3 in the slanted tyrosine supplemented minimal agar media (5 ml agar media in a test tube (1.5×18.5 cm)) adjusted with various pH ranges

Table 1. Effect of temperature on pigment production by *Bacillus licheniformis* SSA3

pH	Temperature			
	Control(20℃)	20℃	30℃	37℃
5.0	−	−	+−	+−
5.5	−	−	+++	+++
6.0	−	−	++++	++++
pH 6.5	−	−	+++++	+++++
7.0	−	+−	++++	++++
7.5	−	+−	+++	+++
8.0	−	+−	+−	+−

The effect of temperature on pigmentation by *Bacillus licheniformis* SSA3 was observed on the solid media adjusted in a wide range of pH and cultured for 5 days without shaking at various temperatures. Abbreviations: −, no pigment; −, yellowish pigment; +, ++, +++, ++++, dark-brown pigment.

(pH5.0-pH8.0) is shown in Table 1. The result showed that pigment synthesis was severely affected at 20℃, but was not affected over 30℃.

We observed that pigment synthesis was completed on solid media within 3-5 days. It seems that during incubation, the difference of temperature between 30℃ and 37℃ did not have much affect on the secondary metabolism of the death phase for pigment synthesis. The pigment synthesis would be initiated by the secondary metabolism after reaching to the death phase as mentioned earlier in this paper.

Cloning of a DNA Fragment involved in Pigment Synthesis

Bacillus licheniformis SSA3 chromosomal DNA was digested with *Hind*III, cloned into the *Hind*III site of pGR71 (6), a promoter detectable vector of *Bacillus*, and then transformed into the unpigmentable *Bacillus licheniformis* SSA3-2MI (7) by protoplast transformation as described in Materials and Methods. Any transformed dark-brown colony was screened on the tyrosine supplemented antibiotic nutrient agar plates, but no dark-brown colony was obtained.

Therefore we digested *Bacillus licheniformis* SSA3 chromosomal DNA partially with *Hind*III. The digested DNA was cloned into the *Hind*III site of pGR71, and transformed by the ligated mixture into the *Bacillus licheniformis* SSA3-2MI by protoplast transformation. The transformed colonies were plated on the tyrosine supplemented antibiotic or control (without antibiotic) nutrient agar plates. Three dark-brown colonies were obtained from the control plates, but no colony from the antibiotic plates.

We prepared mini-prep DNA from the three dark-brown colonies and identified that all three colonies have the same size of self-ligated 7 kb DNA without the vector fragments, designated as pSJ15 (Fig. 5). Fig. 6 showed that pSJ15 could synthesize the dark-brown pigment in the unpigmentable mutant *Bacillus licheniformis* SSA3-

Fig. 5. Photograph of pSJ15.
The size of pSJ15 was 7kb by *EcoRI* digest.

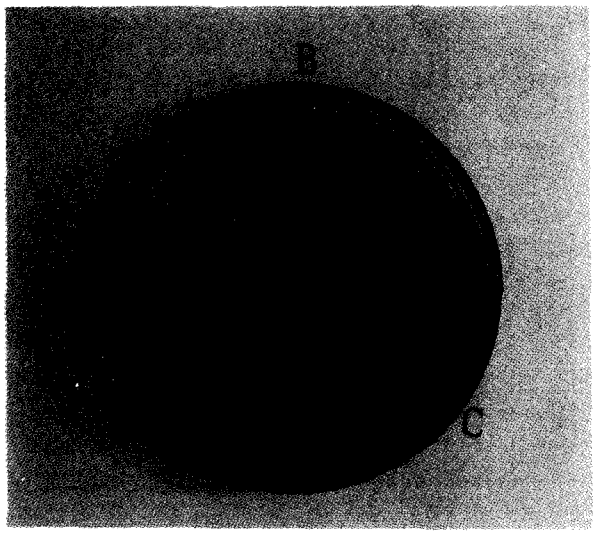

Fig. 6. Pigmentation of pSJ15 in an unpigmentable mutant *Bacillus licheniformis* SSA3-2MI.
Abbreviations: A, wild type *Bacillus licheniformis* SSA3; B, mutant *Bacillus licheniformis* SSA3-2MI; C, *Bacillus licheniformis* SSA3-2MI containing pSJ15. pSJ15 was transferred into the *Bacillus licheniformis* SSA 3-2MI by protoplast transformation as described in Materials and Methods. Plates were incubated for 5 days for pigmentation.

2MI.

Further we transformed pSJ15 into E. coli strain C600 by transformation. The resultant strain could synthesize the dark-brown pigment on the tyrosine supplemented nutrient agar plate as shown in Fig. 7.

The pigmentless mutant, *Bacillus licheniformis* SSA3-2MI was his-, met-, but could synthesize alanine, glycine, tryptophan and tyrosine (7). pSJ15 could synthesize the dark-brown pigment in *Bacillus licheniformis* SSA3-2MI. Therefore we guessed that pSJ15 has certain genes involved in the pigment synthesis beyond tyrosine.

The complementable synthesis of dark-brown pigment by pSJ15 in *E. coli* is very interesting, and may have a

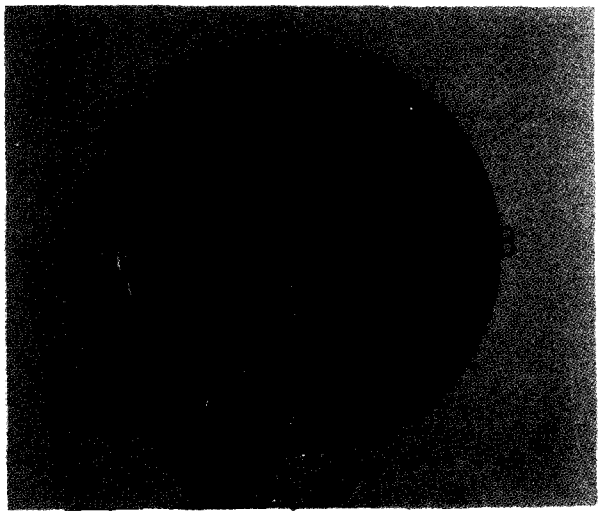

Fig. 7. Pigmentation of pSJ15 in *E. coli* C600. pSJ15 was transferred into C600 by transformation (9).
Abbreviations: A, C600; B, C600 (pSJ15). Plates were incubated for 5 days at 30°C.

gene cluster for this pigment beyond tyrosine.

We are very interested in the genes and genetic structure located on pSJ15. Currently we are sequencing pSJ15.

REFERENCES

1. Barnett, T.A., D. Valenzuela, S. Riner and J.H. Hageman. 1982. Production by *Bacillus subtilis* of brown sporulation-associated pigments. *Can. J. Microbiol.* **29**: 96-101.
2. Barnett, T.A. and J.H. Hagemen. 1983. Chacterization of a brown pigment from *Bacillus subtilis* cultures. *Can. J. Microbiol.* **29:** 309-315.
3. Chang, S. and S.N. Cohen. 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. *Mol. Gen. Genet.* **168:** 111-115.
4. Christans, S. and H. Kaltwasser. 1986. Nickel-content of urease from *Bacillus pasteurii. Arch. Microbiol.* **145:** 51-55.
5. Difco Manual. 1984. *Dehydrated culture media and reagent for microbiology,* 10th ed.
6. Goldford, D.S., R.H. Doi and R.L. Rodrigure. 1981. Expression of Tn9-derived chloramphenicol resistance in *Bacillus subtilis. Nature, 293:* 309-311.
7. Kim, J.K. and S.D. Kim. 1988. Genetic breeding of Korean soy sauce-fermenting Bacillus by UV mutation. *J. Kor. Agr. Chem. Soc.* **31:** 346-350.
8. Kim, J.K., S.M. Park and S.J. Lee, 1995. Novel antimutagenic pigment produced by *Bacillus licheniformis* SSA3. *J. Microbiol. Biotechnol.* **5**:
9. Maniatis, T.E., E.F, Fritsch and J. Sambrook. 1989. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. Scheffer, P. 1969. *Sporulation and the production of* antibiotics, exoenzymes, and extoxins. *Bacteriol. Rev.* **33:** 48-71.

(Received November 17, 1994)