Effects of UV Dose on Formation of Spontaneously Developing Pocks in *Streptomyces azureus* ATCC14921

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Spontaneously developing pocks (S pocks) of *Streptomyces azureus* ATCC14921 were formed by the both functions of conjugative plasmid pSA1 and lysogenic phage SA12. The formation was affected by the dose of UV irradiation. The mean pock diameter in cultures treated with UV light at 0, 7.1, 14.2 and 21.3 × 10^2 μW·erg/cm², respectively, were 1.3, 0.4, 2.2, and 0.5 mm. The dose affected conjugative plasmid pSA1 related to pock formation. There was UV damage of autonomous pSA1 replicon and UV induction of the chromosomal integrated sequence. Increases and decreases in the amount of autonomous pSA1 replicon corresponded to increases and decreases, respectively, in the diameter of the pocks. Both pSA1 and SA12 syntheses were developed in the large pocks (1.3 and 2.2 mm), but only SA12 synthesis was developed in the pinhole pocks (0.4 and 0.5 mm).

**Key words:** *Streptomyces*; spontaneously developing pocks; conjugative plasmid; UV damage; UV induction

Some *Streptomyces* species producing antibiotics form abnormal (or eroded) colonies or lawns with spontaneously developing pocks (S pocks) in solid culture. These pocks appear during the growth of the organisms, and increase in number with repeated transplantsations to solid media. The formation of spores and typical aerial hyphae never occurs in the pocks. S pocks damage bacterial strains being maintained and interfere with the fermentation, as spores are used as a starter for fermentation. S pock formation has been reviewed elsewhere, including measures to control them.1)

S pocks were first noted in *Streptomyces azureus* ATCC149212) and then in *S. hygroscopicus* JCM4213.3) S pocks in thiostrepton-producing *S. azureus* and *S. laurentii* ATCC312554-6) have been investigated in particular detail. The phenomenon seems to be related to another kind of pocks (called C pocks here)7-10) in which conjugative plasmids and their transfer are involved. Both kinds of pocks have circular zones of retarded development of plasmid-accepted transfer. However, there are differences between the pocks: C pocks need a donor strain and a recipient strain for conjugative plasmid transfer, but S pocks occur with a homotypic strain originating from a single spore. A single S pock consisted of a pinhole, the region where aerial and sporulating hyphae were lysed by the lysogenic phage SA12, and a white zone, the region in which the growth of aerial mycelia around the pinhole is retarded by the action of conjugative and integrative plasmid, respectively.1,2) In the center of a C pock, there is a colony formed by the plasmid donor.1)

*S. azureus* ATCC14921 (wild-type strain PK0) harbors a circular conjugative plasmid pSA1 together with a chromosomal integrated sequence, pSA1αβ11,12) After excision, free pSA1 multiplies to 20-30 copies per host genome (unpublished). The free form of the pSA1 replicon is generated from pSA1αβ during development of host mycelia in solid culture, but not in liquid culture.1,2,12) Acidine orange or acriflavin treatment cures the plasmid and inhibits S pock formation.1,2,12) The free form of pSA1 probably is directly involved in the formation of the pocks. Increased numbers of such free forms during serial subculture on the solid media seems to be related to an increased number of S pocks.1,12) However, numbers of pSA1 replicons increase little if at all in liquid culture and in the plasmid-free derivative strains PK100C and PKC, unlike its multicopy derivative pSA1.1,11,12)

The genes of plasmid pSA1 have been sequenced with its derivative pSA1.1 (complete nucleotide sequence has been deposited in databases under accession number AB010724).1,13) Expansion of pSA1 replicon is promoted by the *spi* (kill/trap) (sporulation inhibitory gene: for transfer function in intermycelial plasmid transfer) and *spds* (for spread function in
intramyecial plasmid transfer).\textsuperscript{1,14-16} Plasmid pSA1 also has one set of genes and a sequence for site-specific integration and excision (int, xis, and attP).\textsuperscript{1,12} Excision of pSA1\textsuperscript{wt} probably is catalyzed by the xis (and int) gene products, and it is converted to free form. Excision of pSA1\textsuperscript{wt} may be stimulated by UV irradiation. We have discussed elsewhere the stimulation of excision of pSA1 replicon by UV irradiation.\textsuperscript{1,12} Here, we investigated the effects of the dose of UV on the formation (especially, size and number) of S pocks and on pSA1 replicons in S. azureus.

**Materials and Methods**

**Bacterial strains and medium.** Streptomyces azureus ATCC14921 (wild-type strain PK0) was used. Rye-flakes agar medium (rye flakes, 1%; glucose, 0.2%; yeast extract, 0.1%; CaCO\textsubscript{3}, 0.2%; agar, 1.5%; pH 7.2) was used for mycelial growth and spore production by S. azureus.\textsuperscript{25} The temperature of cultivation was 28°C. Spores were harvested from 7-day-old cultured slants or plates, and spores were prepared as described elsewhere.\textsuperscript{25}

**UV irradiation.** A spore suspension of strain PK0 harvested from an eroded or healthy lawn was irradiated with UV light as follows. First, 5 ml of a spore suspension in a Petri dish 90 mm in diameter was irradiated for 0 to 120 sec at the distance of 300 mm from a UV lamp (GL-15, Toshiba germicidal lamp) with gentle swirling on a turntable. Plates were inoculated with a diluted suspension of spores to give a suitable number of S pocks, colonies, or lawns, and then incubated for 7 days at 28°C. The experiments were done in a dim light.

**Electron microscopy.** Phage preparations were observed with a JEM 2000EX electron microscope as described previously.\textsuperscript{25} Samples taken from a single pock or a normal-looking lawn of mycelia were suspended directly in a drop of potassium phosphotungstate (pH 6.0).

**DNA manipulation.** For extraction and preparation of total DNA of strain PK0, the spores harvested were used to inoculate onto rye flakes agar medium covered with a cellophane membrane, and mycelia were obtained from 4-day-old cultures. Streptomyces DNA was prepared as described by Hopwood et al.\textsuperscript{17} Conventional procedures were used for restriction enzyme digestion and agarose gel electrophoresis.\textsuperscript{18} Southern blotting was done as recommended by manufacturer (Roche Diagnostics; Switzerland).

**Results**

**Effects of UV irradiation on S pock formation**

![Figure 1](attachment:image.png)  
**Fig. 1.** Effect of UV Irradiation on Formation of S Pocks during Subculture. The cultures for each curve were started from 500 normal colonies grown separately in 20 ml vials measuring 27 mm by 55 mm, with a cotton plug so as to form one colony per vial, and then were subcultured every 7 days in the same apparatus with rye-flakes agar medium. ○, Control culture (untreated); ●, culture irradiated with UV light at 14.2 × 10\textsuperscript{2} μW·erg/cm.

Effects of UV irradiation on the appearance of eroded colonies with S pocks from healthy colonies during serial subculture on rye-flakes agar in a preliminary experiment are shown in Fig. 1. UV irradiation at 14.2 × 10\textsuperscript{2} μW·erg/cm resulted in higher proportions of eroded colonies with S pocks than without irradiation. Some 85% of colonies grown from untreated spores were abnormal after seven transplantations, but when colonies were grown from spores irradiated at 14.2 × 10\textsuperscript{2} μW·erg/cm, 85% were abnormal after four transplantations.

**Effect of dose of UV on S pock formation**

When 1.0 × 10\textsuperscript{6} irradiated spores from an eroded lawn together with 1.0 × 10\textsuperscript{6} untreated spores from a healthy lawn (with the healthy spores as well, S pocks were more likely to form) were used to inoculate a plate, the lawn of irradiated spores had peculiar features, compared to the untreated control. Figure 2 shows the effects of various doses of UV on the number of pocks formed. Figure 3 shows changes in shape and size of S pocks with various doses of UV, and Fig. 4 shows a diagram of various S pocks as seen in UV-treated and untreated cultures.

In the lawn of spores irradiated at doses 10.6 × 10\textsuperscript{2} μW·erg/cm or less, the proportion of S pocks was approx. 80 ± 7% (Fig. 2), and the pock diameter was reduced to 0.4 ± 0.1 mm (Fig. 3b, c, and d and Fig 4B); the mean pock diameter in the control lawn was 1.3 ± 0.2 mm (Fig. 3a and Fig. 4A). In pocks of
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In the lawn of spores irradiated at 14.2 and 17.7 × 10² μW·erg/cm, the proportion was 100 ± 4% and 100 ± 6%, respectively (Fig. 2), and the diameter increased to 2.2 ± 0.2 mm at a maximum (Fig. 3e, and Fig. 4C). Pocks in both UV-treated cultures were the same shape. In particular, the white zone was larger than that of untreated controls. All of the pocks consisted of a pinhole in the center and a white zone around the pinhole like control pocks. The mean deviation of size in the enlarged pocks was smaller than that in the controls.

At 21.3 × 10² μW·erg/cm, the proportion of pocks increased to 170 ± 6% (Fig. 2), and the diameter of the pinhole pock increased to 0.5 ± 0.2 mm, but no white zone was observed (Fig. 3f, and Fig. 4D). At 24.8 × 10² μW·erg/cm or more, the number of pinholes seemed fewer (data not shown).

Phage SA12 with defective phage particles was observed in the pinholes of pocks, but rarely in the white zone of pocks, or in the normal lawn of mycelia from UV-treated spores. Whole SA12 (Fig. 5) has a hexagonal head (60 nm in diameter) and a noncontractile tail (210 nm long), as described elsewhere. These results indicate that changes in size and number of S pocks in S. azureus were provoked by UV irradiation.

Effects of UV irradiation on free forms of plasmid pSA1

To examine the relationship between pock size and the amount of the free form of pSA1 replicon, Southern blotting of total DNA prepared from the UV-irradiated solid cultures was done using pSA1.1

Fig. 2. Effects of Dose of UV on Numbers of S Pocks.
The number of spores before UV irradiation was 1.0 × 10⁶. There were 900 ± 20 pocks in the controls. Results are the means of three measurements in three independent experiments.

Fig. 3. Change in Shape and Size of S Pocks Exposed to Various Doses of UV.
a, 0 (control); b, 3.5 × 10² μW·erg/cm; c, 7.1 × 10² μW·erg/cm; d, 10.6 × 10² μW·erg/cm; e, 14.2 × 10² μW·erg/cm; f, 21.3 × 10² μW·erg/cm. Bar, 1 cm.
as probe. The signal of the free form (Fig. 6, arrow A) of pSA1 from the cultures irradiated at 7.1 and 21.3 × 10^2 μW·erg/cm (lanes 3 and 5) was weaker than that in the control culture (lane 2). This difference means that the free form of pSA1 was fewer in number at 7.1 and 21.3 × 10^2 μW·erg/cm. However, at 14.2 × 10^2 μW·erg/cm, the signal (lane 4) was stronger than the control (lane 2). Thus, the free form of pSA1 increased in number at 14.2 × 10^2 μW·erg/cm. The signals at 7.1 and 14.2 × 10^2 μW·erg/cm were almost the same as those seen at 10.6 and 17.7 × 10^2 μW·erg/cm, respectively (data not shown). Arrow C indicates the signal of integrated pSA1 (pSA1<sup>int</sup>) on the chromosomal DNA of strain PK0. The arrow B signal seemed to originate from the replication intermediate of pSA1, because the signals in lanes 2 and 4 were stronger than those in lanes 3 and 5, and were especially strong in lane 4. These results indicated that changes in size and shape of S pocks caused by UV irradiation corresponded to changes in the amount of the free from of pSA1 replicon.

Discussion

The pSA1 replicon is capable of chromosomal integration, excision, and autonomous replication. The formation of S pocks in S. azureus is due to features of the pSA1 replicon. The free form is directly concerned with pock formation, on the basis of following results. A pSA1 replicon-cured strain such as PK100C of S. azureus does not form S pocks. Another pSA1<sup>int</sup>-mutated strain PKC, obtained from a wild-type strain, PK0, by acriflavine treatment, and for which pSA1<sup>int</sup> replicon did not become an autonomous pSA1 replicon, never formed S pocks. Serial transplantation on solid medium causes the excision of pSA1<sup>int</sup>, and increases the number of free pSA1 replicon and the pock formations.

Dose of UV at 14.2 × 10^2 μW·erg/cm increased in the amount of the free form of pSA1, and increased the size of pocks as growth of aerial and sporulating hyphae (white zone) in the pock retarded. The retarded-growth zones (white zones) probably are promoted by the spI/tra gene and spd genes of pSA1, as described above. Expansion of the retarded-growth zones in C pocks is also promoted by the kil/tra gene and spd genes of conjugative pocks.
Reappearance of the white zone with UV treatment presumably is due to newly generated autonomous pSAI replicons and simultaneously from pSAI*rep, because a decrease in the numbers of the free form of pSAI at 7.1×10^6 μW·erg/cm seemed to be related to UV damage to the replication of pSAI. UV induction of autonomous pSAI replicon seemed to occur at the dose of 10.6×10^6 μW·erg/cm, although maximum induction occurred at 14.2×10^6 μW· erg/cm. The simultaneous excision of pSAI*rep in a large number of UV-irradiated spores would be advantageous for transfer of the pSAI replicon to young neighboring mycelia or cells and autonomous replication. For this reason, pocks in cultures irradiated at 14.2 (and 17.7) ×10^6 μW·erg/cm became larger with a white zone, and were more uniform than in control cultures. We concluded that the autonomous pSAI replicon was concerned with expansion of the retarded-growth zone (white zone), but not the formation of pinholes.

Doses of UV at 3.5, 7.1, and 10.6×10^6 μW·erg/ cm reduced the white zone of pocks and slightly decreased the number of pocks and the amount of the free form of pSAI. These results suggest that the replication or transfer function of pSAI replicon was inhibited by UV. However, the decrease in the number of pocks may be due to UV damage in the case of phage SA2 replication. Pinholes were observed in control and UV-treated cultures. In particular, at 21.3×10^6 μW·erg/cm, there were many pinhole pocks, formed presumably by the action of phage SA2, as follows.

The formation of S pocks on strain PK0 is always accompanied by production of a small number of whole particles and many tail-like particles of phage SA2.1-4 This production seems to be involved in S pock formation.4 The induction of SA2 replicon may be concerned with induction of the pSAI replicon. Although pSAI replicon-cured PK100C harbors SA2int, induction of SA2 has not been observed (unpublished data). We presume that excision of SA2int occurs with the co-operation of the pSAI replicon. However, the lytic growth of SA2 develops regardless of the pSAI replicon; many pinholes were observed all over lawns in all cultures examined, and phage SA2 and tail-like particles are found in the pinholes, in which hyphae are lysed.1,2 Excision of SA2int occurs spontaneously and is promoted by appropriate doses of UV, the maximum being 21.3×10^6 μW·erg/cm. Lysis of aerial and sporulating hyphae in the pinhole is due to tail-lysin and endolysin.1,9,20 The purified particles have strong lytic activity toward host mycelia (unpublished data). Active SA21 produced in a very small number, seem to act in the formation of pinholes. Probably, formation of S pocks begins by excision of SA2int with the cooperation of pSAI replicon, and pocks enlarge upon provocation by the autonomous pSAI replicon. Thus, plasmid pSAI in pock formation seems to be escaping from the dying host cell in order to survive. The precise role of phage SA2 on S pock formation will be reported in the near future.

Acknowledgments

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References

13) Yokoyama, E., Matsuzaki, Y., Doi, K., and Ogata, S., Gene encoding a replication initiator protein and


