ASPOROGENOUS MUTANTS OF *BACILLUS SUBTILIS*

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Several asporogenous mutants of *Bacillus subtilis*, which seemed to be one of early sporulating mutants, were obtained by ultraviolet irradiation (strain No. 71 and 7) or acriflavine treatment (strain No. 61). These mutants grew normally in Spizizen's minimal medium and were able to produce amylase, protease, and antibiotics as the wild type in their sporulating stages. Compared with sporulating stage cells of the wild strain, protein degradation during sporulation was of the same degree but protein synthesis did not occur at their sporulating stage in these asporogenous mutants. Therefore, these mutants could not synthesize NADH₂ oxidase, glucose dehydrogenase, and dipicolinate which characteristically appear during sporulation. However, ³H-uracil incorporation experiment during sporulation stage indicated that RNA synthesis occurred at their sporulating stage in these asporogenous mutants.

A number of asporogenous mutants were isolated by many workers to study the mechanism of bacterial sporulation. SCHAEFFER *et al.* showed that the ability to form spores can be restored by transformation in asporogenous mutants of *B. subtilis* (1). SCHAEFFER and IONESCO analyzed several asporogenous mutants and recognized several unlinked genes which control sporulation (2). SPIZIZEN (3) reported that a group of spore genes were clustered with genes for proteolytic enzymes, an antibacterial factor, and a competence factor, by transformation experiment. TAKAHASHI (4-6) obtained many asporogenous mutants of *B. subtilis* and demonstrated, using the transduction experiments, that spore markers of *B. subtilis* are distributed randomly among the chromosomes. Recently, SPIZIZEN (7) also demonstrated by transformation techniques that the defective sites of mutants are scattered and are present in all four linkage groups. With the progress on genetic analysis of sporulation by the use of asporogenous mutants, it is desirable to investigate the defects of these asporogenous mutants by biochemical methods to understand the mechanism of sporulation. We have obtained several asporogenous mutants of *B. subtilis* and investigated the biochemical characteristics of these mutants.

MATERIALS AND METHODS

Bacterial strains. *Bacillus subtilis* MARBURG 111 (leu⁻) strain used throughout this experiment was kindly supplied by Dr. H. Saito, The Institute of Applied Microbiology, University of Tokyo, and was used as sporogenous (sp⁺) strain throughout the experiments. *Staphylococcus aureus* (B-71-1) employed as the test organism for antibiotic production was from The Institute of Applied Microbiology, University of Tokyo.

Chemicals. Acriflavine was purchased from the Tokyo Kasei Kogyo Co., Ltd. (Tokyo). ³H-L-Leucine (200 mCi/mM), ³H-DL-phenylalanine (1590 mCi/mM), and ³H-uracil (5000 mCi/mM) were purchased from the Daiichi Chemical Co., Ltd. (Tokyo). Actinomycin was kindly supplied by Prof. H. Yonehara, The Institute of Applied Microbiology, University of Tokyo.

Media. The following growth and sporulation media were used. L-Leucine (10 µg/ml) supplemented in Spizizen's minimal medium (8) containing per liter of deionized water, glucose 5.0 g, KH₂PO₄ 6.0 g, K₂HPO₄ 14.0 g, sodium citrate·2H₂O 1.0 g, (NH₄)₂SO₄ 2.0 g, and MgSO₄·7H₂O 0.2 g, pH 7.0, was used. Glucose was sterilized by autoclaving separately. In some cases, 0.5% glycerol sterilized separately was used instead of glucose.

Modified SCHAEFFER's medium (9) containing polypepton 5.0 g, meat extract 3.0 g, MgSO₄·7H₂O 0.25 g, KCl 1.0 g, and FeCl₃·6H₂O 10⁻³ mmoles was also used and pH was adjusted to 6.7. CaCl₂·2H₂O 1.0 mmoles and MnCl₂·4H₂O 10⁻² mmoles were added separately to the above medium after autoclaving.

Isolation of asporogenous mutants by ultraviolet irradiation. Cells of *B. subtilis* 111 (leu⁻) grown at the early stationary phase were resuspended in the fresh Schaeffer medium, and were irradiated by a 10-W Mitsubishi Electric germicidal ultraviolet lamp at a distance of 42 cm from the surface of the liquid for 170 sec, and 99.9% killing was effected. After irradiation, the cells were cultured again in the Schaeffer medium at 30° for 12 hr and were then plated on solid Schaeffer medium. Sp⁺ colonies were usually opaque and dark brown while sp⁻ colonies could be detected easily by their translucent appearance after growing for 4–5 days at 40° (1).

Isolation of asporogenous mutants by acriflavine treatment. Experiments were carried out as described by SLEPECKY (10) with a slight modification. *B. subtilis* 111 (leu⁻) was precultured in Spizizen's minimal medium supplemented with 0.002% polypepton for 18 hr at 30°. These cells were then inoculated into the same minimal medium. The inoculum size was approximately 10⁶ viable cells per ml and they were grown with shaking at 45°. Acriflavine was dissolved in deionized water and autoclaved. Acriflavine in a final concentration of 0.6 µg/ml was added to these cell suspensions at 0.5 hr after the initiation of growth. Cells were grown with shaking for 24 hr at 45°. Samples were then diluted and spread on the Schaeffer medium to isolate asporogenous mutants.

Sporulation frequency. To determine the frequency of sporulation, test

strains were grown in Schaeffer's sporulation broth in shaking flasks for 65 hr at 30°. The suspension was diluted appropriately and plated on the Schaeffer agar medium to examine total viability of the population. A second sample was heated at 80° for 30 min, then diluted, and plated on a similar medium to examine spore counts.

Dipicolinate assays. The test strains were grown in the Schaeffer medium for 72 hr at 30°. Then the culture was centrifuged at $5,500 \times g$ for 5 min and separated into cells and supernatant fraction. Dipicolinate content in both fractions was measured by the method of JANSSEN *et al.* (11).

Disruption of cells. The wild strain (No. 111) and asporogenous mutants (No. 61, 71, and 7) were grown in the Schaeffer medium. The cells were harvested from 750 ml of culture liquid by centrifugation at $5,500 \times g$ for 5 min, washed, and resuspended in 15 ml of 0.01 M phosphate buffer (pH 7.4), giving approximately 70 mg/ml (dry weight) cell concentration. The cells were disrupted by a Kubota sonic oscillator at 10 kc for 20 min. The insoluble debris was removed by centrifugation at $15,000 \times g$ for 30 min. The supernatant fraction thus obtained was used as an enzyme preparation. In some cases, this supernatant fraction was centrifuged at $105,000 \times g$ for 3 hr and a particulate fraction was obtained. This particulate fraction was resuspended in 5 ml of 0.01 M phosphate buffer (pH 7.4) and also used as an enzyme preparation. All operations were carried out at 0°.

Protein was estimated according to the procedure of LOWRY *et al.* (12).

Enzyme assays. NADH₂ oxidase was assayed at 30° according to SZULMAJSTER and SCHAEFFER (13). The cuvette contained 0.36 μ moles of NADH₂, 300 μ moles of phosphate (pH 7.4) and 0.1 ml of enzyme, in a total volume of 3.0 ml. The decrease in optical density (OD) at 340 m μ was recorded using a Hitachi spectrophotometer (Model EPS-3). Specific activity was expressed as μ moles NADH₂ oxidized/mg protein/min.

Glucose dehydrogenase was assayed at 30° by a modification of the method of SADOFF *et al.* (15). The cuvette contained 6.0 μ moles of NAD, 0.36 μ moles of NADH₂, 175 μ moles of phosphate (pH 7.4), 300 μ moles of glucose, and 0.1 ml of enzyme in a total volume of 3.0 ml. All the reagents except glucose were mixed first and the reaction was allowed to proceed for 5 min to determine the rate of NADH₂ oxidation by NADH₂ oxidase under these conditions, then glucose was added so that the increase in OD (340 m μ) was detected for NADH₂ production by glucose dehydrogenase. Specific activity was expressed as μ moles NAD reduced/mg protein/min.

Qualitative estimation of various exo-enzymes and antibiotics

Protease production. Test strains were grown on the Schaeffer agar medium, supplemented with 1% casein, for 48 hr at 30°. Then a small volume of 25% trichloroacetic acid was overlaid on these plates. After several minutes, the plates became turbid and protease-forming organisms produced clear zones around the colonies, whereas non-protease-forming mutants did not show such a clear zone.

Amylase production. Test strains were grown on the Schaeffer agar medium supplemented with 1% starch for 48 hr at 30°. Then a small volume of iodine solution was overlaid on these plates. After several minutes, the plates became violet and amylase-forming organisms produced clear zones around the colonies, whereas non-amylase-forming mutants produced no such clear zone.

Antibiotic production. Test strains were grown in the Schaeffer medium with shaking for 30 hr at 30°. The cultures were centrifuged at $5,500 \times g$ for 5 min and the supernatant fractions were obtained. A pulp disc was soaked in the supernatant from each culture and then placed on the Schaeffer agar medium containing a suspension of 10^7 cells per ml of *Staphylococcus aureus*. After 48-hr incubation at 30°, the plates were examined. The antibiotic activity was detected by a large halo around the pulp disc.

Measurement of protein breakdown. Each of test strains was grown in 100 ml of the Schaeffer medium containing 100 μ Ci of ^3H -L-leucine. After 13.5 hr, when sporulation was induced under these conditions, 1.0 mmoles of unlabeled L-leucine was added to the culture. Cell suspension (0.1 ml) was sampled at appropriate time intervals, and then 0.1 ml of cold 10% trichloroacetic acid (TCA) was added to this suspension to precipitate proteins. After 1 hr at 0°, the precipitate formed was placed on glass fiber by filtration and washed twice with cold 5% TCA, once with ethanol-ether (3:2), and finally once with ether. The dried glass fiber was placed in 10 ml of scintillation solution and radioactivity of the acid-precipitable ^3H -leucine was counted in a liquid scintillation spectrometer. Counting efficiency was approximately 8%.

Glass fiber filters (GB-60) were purchased from the Tōyō Roshi Co., Inc. Scintillation fluid consisted of 4.0 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl-POPOP) dissolved in 1 liter of toluene.

Protein synthesis during sporulation. Each strain was grown in 100 ml of the Schaeffer medium. After 13.5 hr, 50 μ Ci of ^3H -DL-phenylalanine was added to the culture. Cell suspension (0.1 ml) was sampled at appropriate time intervals, and radioactivity of the acid-precipitable fraction was counted as described above.

RNA synthesis during sporulation. Each strain was grown in 100 ml of the Schaeffer medium. After 16 hr, when protein synthesis for sporulation occurred in wild strain, 50 μ Ci of ^3H -uracil was added to the culture. Cell suspension (0.1 ml) was sampled at appropriate time intervals, and radioactivity of the acid-precipitable fraction was counted as described above.

RNA content. To measure the RNA content in the cells, 1 ml of cell suspension was sampled periodically. RNA in the cells was extracted by the method of SCHNEIDER (16) and RNA content was measured by the orcinol reaction (17).

Table 1. Frequency of sporulation in wild and asporogenous mutants.
The test strains were grown in Schaeffer sporulation broth for 65 hr at 30°.

Mutant	Viable count (cells/ml)	Spore count (cells/ml)
111 ^a	2.9×10^8	2.7×10^8
61	4.9×10^7	18
71	5.8×10^7	1
7	7.2×10^7	1.8×10^3

^a Wild strain (sp⁺).

RESULTS

Isolation of asporogenous mutant

Two asporogenous mutants (No. 71 and 7) were obtained by ultraviolet irradiation and many asporogenous mutants were also obtained by acriflavine treatment, one of which was named strain No. 61. These mutants were concluded to be originated from the parent wild strain for the following reasons. (1) All mutants retain the leu⁻ marker like the wild strain. (2) Morphological observation of these asporogenous mutants by electron microscopy at the magnification of 5,000 indicates that these mutants are similar to the wild strain. (3) These asporogenous mutants are able to form a spore per 10⁷–10⁸ vegetative cells (Table 1) and these progeny spores also retain the leu⁻ marker.

Frequency of sporulation in asporogenous mutants and wild strain

Frequencies of sporulation in asporogenous mutants and the wild strain were determined (Table 1). Though the wild strain had high sporulation frequency, asporogenous mutant strain No. 61 and No. 71 formed only one spore per 10⁶–10⁷ vegetative cells and asporogenous mutant strain No. 7 formed only one spore per 10³ vegetative cells.

Sporulation frequencies of spores derived from these asporogenous mutants were also determined. The progeny spore of strain No. 61 had the same high frequency as that of the wild strain, but the progeny spores of strains No. 71 and No. 7 had the same low frequency as that of the parental asporogenous mutants.

Growth of asporogenous mutants

The wild strain (No. 111) and asporogenous mutants (No. 61, 71, and 7) were grown in the Schaeffer medium of Spizizen's minimal medium (Figs. 1 and 2). The difference in growth rate between wild strain and asporogenous mutants was not detected at exponential growth phase when the strains were grown in the Schaeffer medium. However, decrease in optical density (660 mμ) caused by cell lysis was observed in asporogenous mutants at the sporulating stage.

Fig. 1. Growth of mutant and wild strains on Schaeffer medium.

○ 111, ■ 61, ▲ 71, ● 7

Fig. 2. Growth of mutants and wild strain on Spizizen minimal medium with glucose or glycerol as a carbon source.

● Glucose, ○ Glycerol

Table 2. Dipicolinate content in wild strain and asporogenous mutants.
Total content of dipicolinate in the wild strain was about 8.1% in dry wt.

72-hr cultivation	Dipicolinate in cells ($\mu\text{g/ml}$)	Dipicolinate in medium ($\mu\text{g/ml}$)
111 sp ⁺	75	22
61 sp ⁻	— ^a	—
71 sp ⁻	—	—
7 sp ⁻	—	—

^a Not detected; sp⁺: wild strain, sp⁻: asporogenous mutants.

Exponential growth rates of wild strain and asporogenous mutants were not significantly different in minimal medium when glycerol or glucose was used as a carbon source. These results suggest that these mutants do not have any metabolic defects with reference to energy metabolism. Therefore, the asporogeneity in these mutants could not be caused by these metabolic defects.

Dipicolinate content and spore-specific enzymes in both wild and mutant strains

The amount of spore-specific component, dipicolinate, was examined in both the wild and mutant strains. As shown in Table 2, the wild strain could synthesize dipicolinate, while dipicolinate was not detected in these asporogenous mutants. These asporogenous mutants failed to form spores even in the presence of 97 $\mu\text{g/ml}$ of dipicolinate in the Schaeffer medium. This result shows that asporogeneity in these mutants could not be caused by the defects in biosynthetic pathway of dipicolinate as reported by HALVORSON *et al.* (18).

Activities of several enzymes such as NADH₂ oxidase and glucose dehydrogenase, which increase significantly during sporulation, were examined in both the wild and mutant strains (13, 14). Extracts were prepared and fractionated from the cells grown in the Schaeffer medium, and NADH₂ oxidase and glucose dehydrogenase activities were measured. As shown in Table 3, these enzymes increased at the sporulating stage six or seven times as much as at the late stationary phase in the wild strain. However, the increase of these two enzymes was not seen at the sporulating stage in asporogenous mutants as shown in Table 4.

Productions of exo-enzymes and antibiotics accompanied with sporulation

The most striking characteristics at the early sporulating stage are the formation and release of antibiotics and of exo-enzymes such as proteases, ribonuclease, and amylase into the growth medium.

The formation of exo-enzymes and antibiotics in these asporogenous mutants was measured to know whether or not these asporogenous mutants were blocked at this early sporulating stage. These asporogenous mutants

Table 3. Increase of enzyme activities during sporulation in wild strain.

Supernatant fraction (15,000×*g*, 30 min) of wild strain 111 was used as enzyme preparations.

	NADH ₂ oxidase (μ moles mg min)	Glucose dehydrogenase (μ moles/mg/min)
O.D. 1.10 (9 hr)	0.018	0.008
O.D. 1.50 (22 hr)	0.103	0.053

Table 4. Activities of NADH₂ oxidase and glucose dehydrogenase in wild and asporogenous mutant strains.

Particulate fraction (105,000×*g*, 3 hr) prepared from the cells of 22-hr cultivation was used as enzyme preparations.

22 hr	NADH ₂ oxidase	Glucose dehydrogenase
111	0.140	0.063
61	0.007	— ^a
71	0.008	—
7	0.011	—

^a Not detected.

Table 5. Exo-enzymes of wild and mutants strains of *B. subtilis*.

	Protease	Amylase	Antibiotic ^a
111	+	+	+
61	+	+	+
71	+	+	+
7	+	+	+

^a Antibiotic activity against *Staph. aureus*.

were able to produce amylase, protease, and antibiotics like the wild strain at the sporulating stage (Table 5).

Breakdown and resynthesis of proteins during sporulation

Previous experiments indicated that the excretion of exo-enzymes and antibiotics at the early sporulating stage occurred in these asporogenous mutants as in the wild strain, but these mutants were not able to synthesize spore-specific components such as dipicolinate, glucose dehydrogenase, and NADH₂ oxidase which appear during sporulation.

It has been demonstrated from a phenomenon of endotrophic sporulation

Fig. 3. Protein degradation during sporulation.

Cells were grown in 100 ml of the Schaeffer medium with 100 μ Ci of ^3H -L-leucine. After 13.5 hr, 1.0 mmole of L-leucine was added to the culture. ○ 111 Sp⁺, ■ 61, ▲ 71, ● 7.

that amino acids needed for the synthesis of new spore-specific proteins can be induced by the breakdown of pre-existing cellular proteins (19). When sporulation was induced in cells, a large part of the vegetative cell proteins was degraded and the resynthesis of protein began so that total protein content remained nearly constant. On the other hand, there was no breakdown of vegetative cell proteins in the protease-less mutant (20). Each strain was grown in 100 ml of the Schaeffer medium containing 100 μ Ci of ^3H -L-leucine. After 13.5 hr, when sporulation was induced under these conditions, 1.0 mmole of unlabeled L-leucine was added to the culture. Cell suspension (0.1 ml) was sampled at appropriate time intervals, and the radioactivity of the acid-precipitable fraction was counted. Fig. 3 shows that there was a breakdown of vegetative cell proteins in both asporogenous mutants and the wild strain with the same degradation rate.

Next, we investigated whether the protein resynthesis occurred during sporulation stage in asporogenous mutants and the wild strain. Each strain was grown in 100 ml of the Schaeffer medium. After 13.5 hr, 50 μ Ci of ^3H -DL-phenylalanine was added to the culture. Cell suspension (0.1 ml) was sampled at appropriate time intervals, and radioactivity of the acid-precipitable fraction was counted.

Protein synthesis during sporulation occurred in the wild strain, while little or no synthesis occurred in these asporogenous mutants as shown in Fig. 4. This might be the reason why these asporogenous mutants were not able to synthesize spore-specific components such as dipicolinate, glucose dehydrogenase, and NADH₂ oxidase at the sporulating stage.

Fig. 4. Incorporation of ^3H -DL-phenylalanine into trichloroacetic acid-insoluble fraction.

^3H -DL-Phenylalanine added after 13.5 hr as indicated by an arrow.

— Absorbance at 660 $m\mu$. ---- Radioactivity.

○ 111, ■ 61, ▲ 71, ● 7

RNA synthesis during sporulation

Our experiments showed that these asporogenous mutants lacked protein synthesis necessary for sporulation, and an examination was made to see whether these asporogenous mutants also lacked RNA synthesis during sporulation. Each strain was grown in 100 ml of the Schaeffer medium. After 16 hr, when protein synthesis for sporulation occurred in the wild strain, 50 μCi of ^3H -uracil was added to the culture. Cell suspension (0.1 ml) was sampled at appropriate time intervals and radioactivity of the acid-precipitable fraction was counted. Although no protein synthesis was detected in asporogenous mutants after 16-hr cultivation, RNA synthesis in these asporogenous mutants continued equally well as the wild strain, as shown in Fig. 5. RNase activity in asporogenous mutants seems to be the same as in the wild strain at the sporulating stage judging from the RNA content in the cells.

Effect of addition of actinomycin on spore formation

It has been reported that net RNA synthesis stops abruptly at stage 1 of sporulation, and total RNA per cell slowly decreases afterwards (21). However, some synthesis of RNA remains at the same period and, therefore, turnover of RNA is seen. Consequently, new kinds of messenger RNA (m-RNA), not produced during vegetative growth, appear early in the sporulation stage (22, 23). Examination was made on the role of RNA synthesis seen after 16-hr cultivation in the Schaeffer medium. Wild strain 111 was grown in 100 ml of the Schaeffer medium, 5 ml of cell suspension was sampled at various growth phases as indicated by arrows in Fig. 6, actinomycin was added to this cell suspension in a concentration of 10 $\mu\text{g}/\text{ml}$, and the cells

Fig. 5. Change in RNA content and incorporation of ^3H -uracil into trichloroacetic acid-insoluble fraction.

^3H -Uracil added after 16 hr as indicated by an arrow.

○—○ Absorbance at 660 $m\mu$. ■—■ Radioactivity.

Fig. 6. Effect of the time of addition of actinomycin on spore formation.

Actinomycin added at the time indicated by arrows and cells were incubated for a total of 48 hr. Control values were 90–95% free spore.

were incubated for 48 hr. The percentage of spores in the culture was determined by plating the culture on the Schaeffer agar medium after heating at 80° for 30 min. Actinomycin (10 µg/ml) inhibited RNA synthesis of *B. subtilis* 111. Fig. 6 indicated that RNA synthesis which was seen around 15–16 hr after the inoculation seemed to be essential for sporulation in the wild strain.

DISCUSSION

The deletion region in these asporogenous mutants seemed to be of a considerable size, as detected by ³H-DL-phenylalanine incorporation experiment during sporulation. In spite of a possibility of large deletion, additional phenotypes were not detected in these mutants, except for the sp⁻ character compared with the wild strain from which the asporogenous mutants were derived.

Recently, IKEDA *et al.* (24) reported that many asporogenous mutants of *B. subtilis* were obtained by high temperature (48°) cultivation. We have also obtained several other asporogenous mutants which were blocked at the early sporulation stage, such as strain Nos. 61, 71, and 7, by various methods including high temperature treatment. Therefore, it was assumed that the asporogenous mutant (strain No. 61) obtained by acriflavine treatment might have been produced by high temperature (45°) cultivation rather than by acriflavine treatment.

No protein synthesis seemed to occur at the sporulating stage in these asporogenous mutants while RNA synthesis seemed to occur normally (Figs. 3 and 4), therefore, it is possible to consider that these mutants have defects at the translation level of protein synthesis for sporulation. However, the possibility that these mutants could not synthesize m-RNA for sporulation cannot be neglected. We have no data yet to clarify these points and the site of defects in spore-specific protein synthesis in asporogenous mutants is still unknown. Preliminary experiment showed that sedimentation patterns of the ribosomes in both the wild strain and the mutants were identical at the sporulating stage.

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