Studies on the Nitrofuran Derivative as a Food Preservative—XV. Microscopic Studies on the Effect of Furfylfuramide on Germination and Outgrowth of Bacterial Spores

Atsushi OBATAKE and Satoshi KARATA*

(Received October 1, 1971)

The effect of furfylfuramide (FF) on germination and outgrowth of two bacterial spores was studied in a slide culture, in order to define the point at which FF acts to prevent the developmental process, from a morphological point of view. Concentrations of FF higher than minimal inhibitory concentration (MIC) did not interfere with the reduction of refractility. However, the developmental process stopped there, and the germinated spores eventually lysed before elongation occurred, shedding the empty spore coats. At less than MIC, many distorted cells appeared and gradually lysed. The bacteriostatic action of FF was shown as a result of the prolongation of their outgrowth. FF acted to arrest the development of bacterial spores not at germination, but after the stage from "swelling" to "emergence". A discrepancy between the two organisms existed in that many refractile spores of *B. coagulans*, isolated from spoiled fish sausage, still remained on the culture after 35 hours at 37°C, but not in the case of *B. subtilis*.

In a previous report,¹⁾ it was described by counting the number of survivals in a medium containing furfylfuramide** (FF) that the drug has shown bactericidal action only at the stage when the spores did not show to be heat resistant. Germination and outgrowth of bacterial spores include five sequential steps: (i) germination (becoming heat-sensitive, nonrefractile and stainable) (ii) swelling of the germinated spore (iii) emergence of the new vegetative cell (iv) elongation (v) cell division.

In order to define the point in developmental process at which FF acts to prevent outgrowth, microscopic observations have been carried out using the slide cultural technique with two kinds of bacterial spore, *i.e.*, a FF sensitive spore and a resistant one isolated from spot type of spoilage of fish sausage. The results of these observations and some discussions are reported in this paper.

Materials and Methods

Organisms used: *Bacillus subtilis* (*B. natto* SAWAMURA) IFO 3010, as a sensitive strain to FF and *B. coagulans*, as a resistant, isolated from spot type of spoilage of fish sausage were employed, as described previously.¹⁾ Spores of these strains were produced on PGY agar (peptone 0.5%, glucose 0.5%, yeast extracts 0.3% and agar 1.5% at pH 7.0),

* Faculty of Agriculture, Kochi Univ., Nankoku, Kochi, Japan (小島 渥・柄多 哲: 高知大学農学部)

** 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide.

incubating at 37°C during 2 weeks for *B. coagulans* and 5 days for *B. subtilis*, in order to harvest a large amount of matured spores. Spore suspensions were prepared according to the aqueous polymer two-phase system described by SACKS and ALDERTON.²⁾

Slide culture: The method described by DUNCAN and FOSTER³⁾ was employed for preparing slide cultures with an exception of incubation temperature, *i.e.*, 37°C for both strains and 43°C only for *B. coagulans*, the latter temperature being the optimum one for *B. coagulans*. Three levels of FF were used in this study: (i) above minimal inhibitory concentration (MIC) ($\times 10$), (ii) about MIC and (iii) below MIC ($\times 1/10$), for two organisms respectively. And the cultures without FF were prepared concurrently as controls. In a preliminary examination, germinative process of the dormant spore had been comparatively studied with several media: PGY agar, Casamino acid agar⁴⁾ and nutrient agar. Then we decided to use PGY agar for the slide culture, since germinative processes were not different in these media and suitability of bright field of vision for photomicroscopy was gained with that medium.

Results

Spores of both strains employed in controls showed normal morphogenic transformation from dormant spores to vegetative cells as diagrammatically outlined in Fig. 1. Microscopic observations were roughly sketched in diagrammatical illustrations in Fig. 2 being based on the microphotographs as presented in Plates 1–2.

Fig. 1. Diagrammatical outline of normal morphogenic transformation from dormant spore to vegetative cells.

In the case of *B. subtilis*, elongation occurred within 2 hours incubation at control and 0.01 γ /ml FF levels, and then at the former successfully developed into cell division after 6 hours, whereas at the latter many distorted cells appeared and failed to develop, so that the duration for multiplication from germinated spore at 0.01 γ /ml level was longer than that of the control. At FF concentrations of 0.1 γ /ml and 1 γ /ml, most of the refractile spores germinated after 2 hours incubation, but even after 24 hours any elongated cells

did not appear at all. The cells that did not develop beyond the elongation stage eventually lysed, leaving the empty spore coats.

In *B. coagulans*, the elongated cells and cell division were observed after 5 hours and 10 hours incubation respectively at less than 0.1 γ /ml FF levels. At 1 γ /ml FF level, which may be considered as inhibiting concentration of growth for this organism, many abnormal cells appeared and failed to develop into normal vegetative cells. Then, the

Fig. 2. Diagrammatical illustration of phase-contrast microscopic observation on the slide cultures of bacterial spores and vegetative cells containing several concentrations of FF ordiante: concentration of FF in γ /ml
abscissa: incubation time in hours
(A) Spores of *B. subtilis*
(B) Spores of *B. coagulans*
(C) Vegetative cells of *B. subtilis*

duration for development to cell division from refractile spore required about 35 hours. At 10 γ /ml FF level, germinated spores appeared after 5 hours, but elongation was completely blocked during the whole 35 hours incubation. The germinated cells were eventually lysed, shedding the spore coats. The result was the same as that obtained from *B. subtilis* at 1 γ /ml FF. It was, however, different from the case of *B. subtilis* that some refractile, not germinated, spores of this strain still remained on the culture till 35 hours incubation. Since this might have been caused by incubation temperature lower than the optimum temperature, the same experiment was carried out at 43°C, which was optimum for this strain. Almost same results were obtained except for the shorter duration of the developmental process.

Additional experiments were carried out at 3 γ /ml and 5 γ /ml FF levels for *B. coagulans*. As the results obtained were similar to that from 10 γ /ml FF, it was suggested that MIC against this strain was somewhere around 3 γ /ml FF level.

Next, experiments with slide cultures for vegetative cells obtained from preliminary cultures were also carried out, for the purpose of estimating whether FF acted only at the stage of germination or not. For *B. subtilis*, after 15 hours incubation, at less than 0.01 γ /ml vegetative cells multiplied, whereas at more than 0.1 γ /ml, they did not divided and

then lysed. In the case of *B. coagulans*, after 12 hours incubation, the multiplication appeared in the control, but at more than 1 γ /ml vegetative cells also lysed. These results reveal that FF may also have acted on the vegetative cells at the same concentration as that for the bacterial spores in both *B. subtilis* and *B. coagulans*.

Discussion

With a slide cultural technique, GOULD⁵⁾ has already investigated the effect of many preservatives, not including nitrofurantoin derivatives, on germination of bacterial spores. He reported that germination was always more resistant than later growth from the spore, and could be prevented by using concentrations of preservatives greater than the minimum required to inhibit a particular stage of growth.

In this study using a nitrofurantoin derivative, even higher concentration of FF, ten times as much as MIC for both strains employed, did not interfere with the reduction of refractility without any temporal delay compared with the control. However, the process stopped there, so that the germinated spores eventually lysed before elongation occurred, leaving the empty spore coats. At less than MIC of FF, many distorted cells appeared and gradually lysed. It, therefore, required a long time for the normal cells remaining on the culture to multiply collectively. It is suggested that the bacteriostatic action of FF was shown as the result of this prolongation of outgrowth.

From the facts described above, it may be concluded from a morphological point of view that the point, at which FF acts to arrest the developmental process of bacterial spores, is in existence after the stage from "swelling" to "emergence", not in germination.

The present results coincide with the description of PAUL and PAUL⁶⁾ in suggesting that the developmental stage most sensitive to the interference of nitrofurantoin derivatives appears to be that just prior to the stage at which certain cell divisions would normally take place. It is, however, suggested that FF acted not only at the stage of the outgrowth, but at that of cell division, since it also inhibited the multiplication from vegetative cells at the same level as for bacterial spores.

It required a longer incubation period for *B. coagulans* to germinate in the control than *B. subtilis*. Many refractile spores of *B. coagulans* still remained on the slide culture containing FF at 10 γ /ml till 35 hours incubation at 37°C, and till 30 hours at 43°C. It can not be considered that this marked discrepancy between two organisms used in this study is due to the medium employed, since the same results were obtained in different media: PGY medium supplement with 50 mM L-alanine, one proposed by DONNELAN *et al.*⁴⁾, and a casamino acid medium⁴⁾.

In any case, it is worth noticing that the refractile spore of *B. coagulans*, which was isolated from a fish sausage as an organism causing the spot type of spoilage, considerably

remained till 35 hours incubation in this study, considering that this type of spoilage appears when FF does not remain in fish sausage,⁷⁾ and that FF shows bactericidal action only at the stage when bacterial spores were heat susceptible.¹⁾

References

- 1) A. OBATAKE and T. MATSUDA: This Bull., **35**, 1131-1137 (1969).
 - 2) L. E. SACKS and G. ALDERTON: *J. Bacteriol.*, **82**, 331-341 (1961).
 - 3) C. L. DUNCAN and E. M. FOSTER: *Appl. Microbiol.*, **16**, 406-411 (1968).
 - 4) J. E. DONNELLAN, E. H. NAGS, and H. S. LEVINSON: *J. Bacteriol.*, **87**, 332-336 (1964).
 - 5) G. W. GOULD: in "Microbial Inhibitors in Food" (N. MOLIN, ed.), 17-24, Almqvist & Wiksell, Stockholm (1964).
 - 6) H. E. PAUL and M. F. PAUL: The Nitrofurans—Chemotherapeutic Properties, in "Experimental Chemotherapy II", 345-356, Academic Press, London (1964).
 - 7) A. OBATAKE: This Bull., **31**, 370-376 (1965).
-

Explanation of the Plates

Plate 1. Microphotographs of germination and outgrowth of *B. coagulans* spores on slide cultures containing FF at 37°C at different incubation periods. $\times 600$

Plate 2-1. The same as Plate 1, for spores of *B. subtilis*

Plate 2-2. Microphotographs of bacterial growth on slide cultures of the vegetative cells of *B. subtilis* containing FF at 37°C. $\times 600$

Plate 2-3. The same as Plate 2-2, for vegetative cells of *B. coagulans*.

The numbers indicate the concentration of FF in γ /ml and the incubation time in hours. Thus, 0.1-15 indicates 15 hours after incubation at 0.1 γ /ml FF level.

Plate 1.

Plate 2-1.

Plate 2-2.

Plate 2-3.