Spore Production by Bacillus stearothermophilus

JUHEE KIM AND H. BROOKS NAYLOR

Department of Dairy and Food Science, New York State College of Agriculture,
Cornell University, Ithaca, New York

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Cultures of B. stearothermophilus included in this study were: ATCC 7953, ATCC 7954, ATCC 10149, and one strain isolated in our laboratory. The medium used for preparation of the inoculum contained Tryptone, 1%; yeast extract (Difco), 0.5%; K₂HPO₄, 0.2%; and was adjusted to pH 7.2 before being sterilized by autoclaving. This presporulation medium will be referred to as TYG broth in this report. Inocula produced in TYG medium grew and sporulated much better when transferred to the sporulation medium than did inocula prepared in nutrient broth. The sporulation medium contained nutrient broth (Difco), 0.8%; yeast extract (Difco), 0.4%; MnCl₂·4H₂O, 10 ppm; Difco agar, 2.0%; and was adjusted to pH 7.2. It was found that spore yields were drastically reduced when the yeast extract concentration was lowered. The use of 2% agar rather than the usual 1.5% improved moisture retention during incubation at high temperature, and facilitated washing of the spores from the agar surface.

The method employed for the production of spores is shown schematically in Fig. 1. The inoculation sequence was initiated by transferring a loop of stock culture (sporulated cells grown on a slant of sporulation agar and stored in a refrigerator) to a test tube containing 5 to 7 ml of TYG broth, and incubating at 52 to 53 C on a New Brunswick rotary shaker for 12 to 14 hr.

A 2-ml amount of this young culture was spread onto the surface of a solidified sporulation agar (80 to 100 ml) contained in a large glass petri dish (150-mm diameter). The plate was incubated in an upright position at 52 to 53 C until no free liquid was present on the agar surface (about 10 hr). The plate was then inverted to minimize dehydration of the agar, and the incubation was continued until 80 to 90% of the cells had sporulated as determined by microscopic examination. This occurred between 18 and 26 hr after inversion of the plate. The spores from this plate were then used as the inoculum for preparing larger quantities of spores by carefully washing the agar surface with 20 ml of TYG broth and adding it to 80 ml of TYG medium contained in a 2-liter indented Erlenmeyer flask. After incubating the flask on a rotary shaker for 12 to 14 hr at 52 to 53 C, the culture was used to inoculate 40 large petri plates, containing sporulation agar, at the rate of 2 ml of culture per plate. The plates were then incubated as described above. After sporulation occurred, the plates were cooled to room temperature, and the spores were washed.

FIG. 1. Inoculation sequence of spore production by Bacillus stearothermophilus.
off with ice-cold distilled water. The washings from each plate were transferred quickly to a flask immersed in crushed ice. The resultant spore suspension was centrifuged at 1 C to sediment the spores. After decanting the supernatant fluid, an equal volume of cold distilled water was added to resuspend the sediment. Lysozyme was added as a 1% solution at the rate of 1 ml per 100 ml of spore suspension. Overnight incubation at 1 to 3 C assured liberation of all spores from sporangia, and lysis of any unsporulated cells in the preparation. After the lysozyme treatment, the spores were washed repeatedly by centrifugation at 1 C with the use of ice water to resuspend the sedimented spores.

All four strains of *B. stearothermophilus* tested gave excellent yields of spores with the procedure described above.
Antibiotic Resistance in *Mycoplasma* Isolates from Tissue Cultures

SHARON B. RAHMAN, JOAN B. SEMAR, AND D. PERLMAN

*Squibb Institute for Medical Research, New Brunswick, New Jersey 08902*

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During the past few years there have been a number of reports of problems encountered in controlling *Mycoplasma* infections in tissue cultures. The usefulness of a number of antibiotics has been reported (T. R. Carski and C. C. Shepard, J. Bacteriol. 81:626, 1961; P. Balduzzi and R. J. Charbonneau, Experientia 20:651, 1964; D. Perlman, S. B. Rahman, and J. B. Semar, Appl. Microbiol. 15:82, 1967), and some investigators have advocated routine use of kanamycin, tylosin, or tetracyclines to control chance contaminations. We examined the antibiotic susceptibility patterns of *Mycoplasma* isolated from six tissue cultures and found that only two of the group were susceptible to these antibiotics.

These *Mycoplasma* strains were isolated from the tissue cultures by an adaptation of Pollock's (*personal communication*) method, by use of soypeptone-agar containing agamma human serum. Agar blocks containing the typical *Mycoplasma* colonies were transferred to tubes of soypeptone broth (containing the agamma human serum), and the tubes were incubated at 37 C in a 5% CO₂-95% air atmosphere for 3 or 4 days. These suspension cultures were used as inoculum source for antibiotic susceptibility tests.

The antibody susceptibility was determined by streaking the *Mycoplasma* suspensions on soypeptone agar (with agamma human serum) containing the test antibiotics. The plates were incubated for 5 to 7 days at 37 C in the CO₂-air incubator, and growth was noted by microscopic inspection.

Only two of the six isolates were inhibited by tylosin, kanamycin, and tetracycline (each at 20 ppm). Lincomycin and spiramycin (each at 20 ppm) inhibited the four isolates resistant to tylosin, kanamycin (200 ppm), and tetracycline; several of these isolates were also susceptible to paromomycin (200 ppm) and to gentamicin. An antiserum to one strain, prepared by the method of Pollock (Proc. Soc. Exptl. Biol. Med. 112:176, 1963), did not react with one of two *Mycoplasma* isolates tested, and thus had limited use in our program.

These results lead us to conclude that *Mycoplasma* isolates from contaminated tissue cultures may vary widely in their antibiotic susceptibility patterns, and it is likely that no one antibiotic is available which will inhibit all *Mycoplasma* strains. Our previous study (D. Perlman et al., Appl. Microbiol. 15:82, 1967) showed that antibiotic resistance can be rather easily induced in *Mycoplasma*, and we cautioned against indiscriminate use or continued use of a variety of antibiotics in tissue culture media as a prophylactic measure. Such practice might result in development of *Mycoplasma* strains which would have resistance to all of the noncytotoxic antibiotics, and then there might be no easy way to control them.

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*Department of Dairy and Food Science, New York State College of Agriculture, Cornell University, Ithaca, New York*

Volume 14, no. 4, page 690, first column, 4 lines from bottom of page: Change "The medium used for preparation of the inoculum contained Tryptone, 1%; yeast extract (Difco), 0.5%; K₂HPO₄, 0.2%; and was adjusted to pH 7.2 before being sterilized by autoclaving." to "The medium used for preparation of the inoculum contained Tryptone, 1%; yeast extract (Difco), 0.5%; glucose 0.5%; K₂HPO₄, 0.2%; and was adjusted to pH 7.2 before being sterilized by autoclaving."