Requirement for and Sensitivity to Lysozyme by Clostridium perfringens Spores Heated at Ultrahigh Temperatures

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The requirement of ultrahigh temperature (UHT)-treated Clostridium perfringens spores for lysozyme and the sensitivity of heated and unheated spores to lysozyme were studied. The UHT-treated spores requiring lysozyme for germination and colony formation originated from only a small portion of the non-UHT-treated spore population. This raised a question of whether the requirement for lysozyme was natural to the spores or was induced by the UHT treatments. However, these spores did not require lysozyme for germination before UHT treatment, which confirmed that the requirement for lysozyme had been induced by the UHT treatment. Only 1 to 2% of the spores were naturally sensitive to lysozyme; therefore, the mere addition of lysozyme to the plating medium did not permit the enumeration of all survivors. Treatment of UHT-treated spores with ethylenediaminetetraacetate (EDTA) sensitized the spores to lysozyme and increased by 10- to 100-fold the number of survivors that were detected on a medium containing lysozyme. Under the heating conditions used, spores that were naturally sensitive to lysozyme and spores that required EDTA treatment were equally heat resistant.

The recovery of severely heated Clostridium perfringens spores was greatly improved if the enumeration medium was supplemented with lysozyme (1, 3, 4). Lysozyme did not increase the colony counts of heat-activated spores, which suggested that the requirement for lysozyme was not natural to the spores but had been induced by the heat treatments (injury). For C. perfringens spores heated at ultrahigh temperatures (UHT), however, the spores responding to lysozyme in the recovery medium were derived from only a small portion (1 to 2%) of the non-UHT-treated spore population (1). Had these spores naturally required lysozyme for germination, a 1 to 2% difference between the colony counts of heat-activated spores enumerated on a medium with or without lysozyme would not have been detected by the agar plate count method (plating duplicate samples). It was not certain, therefore, that the spores had actually been injured by UHT treatment.

When UHT-treated C. perfringens spores were enumerated on a medium containing lysozyme, the time-survivor curves were biphasic-concave (1). Such curves are typically observed for the inactivation of spore populations composed of two types of spores. The biphasic time-survivor curves were observed only when lysozyme was present in the medium and when lysozyme germinates C. perfringens spores (3, 4), which suggested that the two types of spores differed in their sensitivity to lysozyme or in the heat resistance of their outgrowth systems. The distinction is vital to the development of thermal processes capable of lowering to an acceptable level the number of viable C. perfringens spores in a food. Unless all injured survivors are sensitive to lysozyme, the mere addition of lysozyme to the recovery medium is inadequate for their detection.

The findings presented here show that: (i) the requirement for lysozyme was induced by heating; (ii) within a spore population, the spores differed in their sensitivity to lysozyme; and (iii) enumeration of all survivors required that the heated spores be sensitized to lysozyme prior to enumeration on a medium containing lysozyme.

MATERIALS AND METHODS

Maintenance of test organisms, composition and preparation of media, preparation of spore suspensions, heat treatments, and methods for enumeration of surviving spores have been described (1).

Germination of lysozyme-sensitive spores in a complex medium. An aqueous suspension (10⁶ spores/ml) of C. perfringens NCTC 8798 spores was
heated at 75°C for 20 min to activate the spores and was then divided into two portions. Portion A was held in ice. Portion B was centrifuged at 12,100 × g for 20 min, the supernatant was removed, and the spores were suspended in Trypticase (15 g/liter)-yeast extract (10 g/liter) broth (TYB). This medium was similar to the Trypticase-yeast extract-citrate-sulfite agar (TYCS) plating medium used for the enumeration of survivors (1). The spores were held at 35°C for 1 h, heated at 75°C for 20 min to kill germinated spores, washed twice with water, and resuspended to the original volume. Both portions were heated at 105°C in capillary tubes, and survivors were enumerated on TYCS plus lysozyme (18 U/ml) (Sigma Chemical Co., St. Louis, Mo.).

**Germination of lysozyme-sensitive spores by lysozyme.** Non-heat-activated strain 8798 spores were suspended in 50 mM sodium phosphate buffer (pH 7) with or without 18 U of lysozyme per ml. The spores suspended in each medium were incubated at 45°C for 1 h and then heated at 75°C for 20 min to kill any germinated spores and heat activate the ungerminated spores. The spores were then washed twice, suspended in distilled water, heated at 105°C, and enumerated on TYCS plus lysozyme (18 U/ml).

**EDTA sensitization of UHT-treated spores.** Duplicate capillary tubes, each containing 0.05 ml of UHT-treated spore suspension, were crushed in 100 ml of 10 mM sodium ethylenediaminetetraacetate (EDTA; pH 9.5) and held at 45°C for 1 h (2). The spores then were diluted in 0.1% peptone water (plus 0.02% Antifoam B, Sigma Chemical Co. St. Louis, Mo.) and enumerated on TYCS with or without lysozyme (18 U/ml).

**RESULTS AND DISCUSSION**

**Injury during UHT treatment.** We reported (1) that UHT-treated *C. perfringens* spores requiring lysozyme for colony formation appeared to derive from only 1 to 2% of the non-UHT-treated spore population. This portion of the total population was so small that it was not certain if the requirement for lysozyme was induced by UHT treatment (injury) or was natural to the spores. If these spores naturally required lysozyme for germination and colony formation, a medium containing lysozyme should be effective for the enumeration of all survivors. However, if these spores were injured during UHT treatment, the remaining spores also may have been injured but were not detected on TYCS plus lysozyme. The mere incorporation of lysozyme in the enumeration medium may not be sufficient for the detection of all survivors.

To determine whether spores requiring lysozyme after UHT treatment were able to germinate normally before UHT treatment, heat-activated strain 8798 spores were incubated in TYB, heated to kill germinated spores, and then UHT treated. Spores incubated in water instead of TYB were treated similarly. Colony counts of non-UHT-treated spores incubated in water or TYB were 1.3 × 10⁶/ml and 7 × 10⁷/ml, respectively, indicating that 94% of the spores had germinated in TYB (Fig. 1). If 1 to 2% of the spores had been unable to germinate normally in TYB (i.e., required lysozyme for germination), they should constitute 17 to 33% of the spores remaining after this extensive germination. This change in the composition of the spore population would be reflected in the time-survivor curves, because only the second phase of the curves represented a response to lysozyme (1). However, the time-survivor curves for spores held in TYB or water were identical, indicating that the relative composition of the spore suspension was unchanged by germination in TYB. The spores that required lysozyme after UHT treatment had been able to germinate normally before UHT treatment.

![Fig. 1. Influence of prior germination in Trypticase-yeast extract broth on the inactivation kinetics of *Clostridium perfringens* NCTC 8798 spores recovered on TYCS plus lysozyme (18 U/ml).](http://aem.asm.org/)
This confirmed that the spores had been injured by the UHT treatments and suggested that other spores also may have been injured but were not detected on TYCS plus lysozyme.

**Basis for the biphasic time-survivor curves.** The time-survivor curves were biphasic-concave only when lysozyme was used in the enumeration medium (1). Lysozyme reportedly germinated injured _C. perfringens_ spores (3, 4). If lysozyme germinated only some of the spores, the biphasic survivor curves must represent the rapid injury of spores not sensitive to lysozyme and the slower inactivation of the outgrowth system of spores sensitive to lysozyme. Alternatively, if all of the spores are sensitive to lysozyme, the two portions of the survivor curves reflect inactivation of the outgrowth systems in spores that differ in heat resistance. In the latter case, many of the heated spores germinated by lysozyme would not complete outgrowth, and the number of spores germinated by lysozyme should be up to 100-fold greater than the number of survivors enumerated on a medium containing lysozyme. To test this, strain 8798 spores were heat activated and then UHT treated at 105 C for 2.5 or 5 min. Survival and germination activity of non-UHT-treated and UHT-treated spores were determined. The data in Table 1 show that UHT treatment greatly reduced the number of spores able to germinate in TYB. Germination of UHT-treated spores in TYB plus lysozyme, however, was no greater than in TYB without lysozyme, and the extent of germination in either medium was similar to the level of survival measured on TYCS plus lysozyme. This indicated that the majority of the UHT-treated spores were not sensitive to lysozyme and suggested that the biphasic nature of the time-survivor curves was based on differences among the spores in their sensitivity to lysozyme rather than on differences in the heat resistance of their outgrowth systems.

Results of an earlier study (1) suggested that a portion of strain 8798 spores was naturally sensitive to lysozyme. The spores required heat activation for normal germination, and germination of a small percentage of the spores by lysozyme was indicated by the higher colony counts of non-heat-activated spores on TYCS with lysozyme than on TYCS without lysozyme. The experiment was repeated with 10 replicates for each medium so that the results could be statistically analyzed. In the absence of lysozyme, 3.7% of the spores formed colonies; with lysozyme in the medium, 5.9% of the spores germinated and formed colonies. This increase of 2.2 percentage units was statistically significant of _P_ = 0.025 ( _t_ test, degrees of freedom = 13) and was similar in magnitude to the percentage of heat-activated spores that had appeared to be unique in their sensitivity to lysozyme or the heat resistance of their outgrowth systems (1).

In another experiment, unheated strain 8798 spores were incubated in a lysozyme-phosphate buffer mixture to allow the germination of any

![Graph](http://aem.asm.org/)

**FIG. 2. Influence of exposure of non-heat-activated *Clostridium perfringens* NCTC 8798 spores to lysozyme on subsequent inactivation kinetics and recovery on TYCS plus lysozyme (18 U/ml).**

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**Table 1. Germination and survival of UHT-treated *C. perfringens* NCTC 8798 spores**

| Heat treatment          | Germination* (%) | Survivors* (%) |
|-------------------------|------------------|----------------|
|                         | TYB | TYB + lysozyme |                  |
| 75 C/20 min             | 93  | 93            | 100              |
| 75 C/20 min + 105 C/2.5 min | 10.2 | 12.2          | 14               |
| 75 C/20 min + 105 C/5 min | 0.0  | 0.0           | 2.6              |

*Measured as the decrease in optical density at 650 nm after 1 h at 35 C.

*Measured on TYCS plus 18 U of lysozyme per ml.

*TYB contained 50 µg of chloramphenical per ml.
lysozyme-sensitive spores prior to heat activation and UHT treatment. Spores held in phosphate buffer without lysozyme served as a control. The lysozyme pretreatment did not affect colony counts of heat activated-non-UHT-treated spores (data not presented) or the inactivation kinetics of spores not responding to lysozyme in the plating medium (represented by the first phase of the survivor curves, Fig. 2). However, for lysozyme-pretreated spores, the first phase of the survivor curve extended to a lower level of survivors before the response to lysozyme in TYCS (indicated by the second phase of the time-survivor curve) was observed. Extrapolation of the second phase of each survivor curve indicated that 97% of the spores that normally would have been germinated by lysozyme in TYCS already had been germinated by lysozyme during pretreatment. This confirmed that a small portion of the unheated spores was naturally sensitive to lysozyme, and suggested that it was recovery of the surviving fraction of these spores that was enhanced by the use of lysozyme in TYCS.

Generally, bacterial spores are resistant to lysozyme and require some sensitization treatment (5). Bacillus megaterium ATCC 9885 spores appear to be the only exception (6). However, natural sensitivity to lysozyme by a very small portion of a spore population, as shown here for C. perfringens NCTC 8798 spores, would be easily overlooked. The natural sensitivity of B. megaterium ATCC 9885 spores may not be as unique as the large percentage of the spores having this trait.

To confirm that the biphasic time-survivor curves reflected differences among the spores in their sensitivity to lysozyme, UHT-treated strain 8798 spores were incubated in EDTA prior to enumeration of survivors. Unheated C. perfringens spores were sensitized lysozyme by EDTA (2). The EDTA treatment did not influence the recovery of spores on TYCS lacking lysozyme, but increased by up to 100-fold the number of survivors detected on TYCS plus lysozyme (Fig. 3). This was observed for strain 8798 spores heated at 105 or 120 C (Fig. 3) and for two other strains of C. perfringens spores (Table 2). The time-survivor curves for EDTA-treated spores enumerated on TYCS plus lysozyme were linear and unbroken, which indicated that all of the spores had been sensitized to lysozyme and that the biphasic nature of the time-survivor curves resulted from the resistance of many of the survivors to lysozyme. When survivors were enumerated on TYCS plus lysozyme, the D values (decimal reduction times: times required for a 90% decrease in the number of viable spores) for spores requiring EDTA treatment were similar to those for spores naturally sensitive to lysozyme. This indicated that, for the particular heating conditions used, the two types of spores were equally heat resistant.

The results demonstrate that many of the C. perfringens spores surviving UHT treatment were injured and required lysozyme for germination and colony formation, but the majority were not sensitive to lysozyme; maximal recovery of survivors required that the heated spores be sensitized to lysozyme prior to enumeration. In the absence of such a treatment, more than 90% of the survivors were not detected, and the actual heat resistance of these spores was unknown.

The usefulness of lysozyme for the recovery of injured spores may not be limited to C. perfringens spores. Alderton, Chen, and Ito (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 17, 1973) reported that lysozyme increased the recovery of heated C. botulinum spores. As with C. per-

| Strain     | Heat treatment | Survival (%) |
|------------|----------------|--------------|
|            |                | -EDTA* | +EDTA |
| 8798       | 105 C for 10 min | 0.20 | 11 |
| 8238       | 115 C for 15 s    | 0.20 | 2.3 |
| 10240      | 100 C for 60 s    | 0.16 | 1.5 |

*With (+) or without (-)* EDTA treatment of heated spores prior to plating on TYCS plus 18 U of lysozyme per ml.

![Fig. 3. Influence of EDTA treatment of heated Clostridium perfringens NCTC 8798 spores on the recovery of survivors on TYCS and TYCS plus lysozyme.](http://aem.asm.org/Downloaded from http://aem.asm.org/)
fringens spores, however, the time-survivor curves were linear and unbroken when survivors were enumerated on a medium lacking lysozyme, but were biphasic-concave when lysozyme was used in the medium. This suggests that the effective use of lysozyme for the enumeration of injured C. botulinum spores, or spores of other species, may require that the spores be sensitized to lysozyme before the survivors are enumerated.

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LITERATURE CITED

1. Adams, D. M. 1973. Inactivation of Clostridium perfringens type A spores at ultrahigh temperatures. Appl. Microbiol. 26:282-287.
2. Adams, D. M. 1973. Sensitization by ethylenediaminetetraacetate of Clostridium perfringens type A spores to germination by lysozyme. J. Bacteriol. 116:500-502.
3. Cassier, M., and M. Sebald. 1969. Germination lysozyme dépendante des spores de Clostridium perfringens ATCC 3624 après traitement thermique. Ann. Inst. Pasteur (Paris) 117:312-324.
4. Duncan, C. L., R. G. Labbe, and R. R. Reich. 1972. Germination of heat- and alkali-altered spores of Clostridium perfringens type A by lysozyme and an initiation protein. J. Bacteriol. 109:550-559.
5. Gould, G. W., and A. Hurst. 1969. The bacterial spore. Academic Press Inc., New York.
6. Suzuki, Y., and L. J. Rode. 1969. Effect of lysozyme on resting spores of Bacillus megaterium. J. Bacteriol. 98:238-245.