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Action of Egg White Lysozyme on *Clostridium tyrobutyricum*

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A 500-U ml^{-1} portion of egg white lysozyme was able to kill 99% of 5×10^5 resting vegetative cells of *Clostridium tyrobutyricum* within 24 h of incubation at 25°C. Spores were completely resistant to lysozyme. Proliferating vegetative cells were severely inhibited, although lysozyme-resistant cells developed in growing cultures in the presence of lysozyme. Whereas early stages of spore germination (loss of optical refractility and heat resistance) were not inhibited by lysozyme, the overall outgrowth of spore cells into vegetative cells was delayed by 1 day in the presence of 500 U of lysozyme ml^{-1}. This delay was independent of the lysozyme sensitivity or resistance of the mother culture of the used spores. It is suggested that this inhibition by lysozyme of the outgrowth of spore cells into vegetative cells of the lactate-fermenting *C. tyrobutyricum* is the basis for the observation that lysozyme can substitute for nitrate in preventing the "late gas" defect of Edam- and Gouda-type cheeses.

Contamination of cheese milk with spores of lactate-fermenting clostridia, especially *Clostridium tyrobutyricum*, originates mainly from the use of silage in the feeding of dairy cattle. Since these spores easily survive pasteurization temperatures, they provoke a typical defect called "late gas" (3) during the ripening of cheeses of the Edam and Gouda type. Addition of nitrate to the cheese milk is a common and successful practice used to prevent the late gas defect, which otherwise yields an unacceptable product. The active compound inhibiting germination of these spores is nitrite produced from nitrate by microbiological or enzymatic reduction, or both (7). Nitrite, in turn, may give rise to the formation of nitrosamines in foods rich in organic secondary amines. Although the incidence and amounts of nitrosamines in cheese produced with nitrate are extremely low (2), substitution of nitrate in cheese production may lower unwanted consumption of nitrate and nitrite. In addition, any system capable of inhibiting the outgrowth of clostridial spores, including *Clostridium botulinum* spores, in foods could provide an important model for lowering the risk of nitrate consumption. Egg white lysozyme has been shown to prevent the late gas defect of Edam cheese completely even if spores derived from lysozyme-resistant vegetative cells have been used to contaminate the cheese milk (6). Our main concern has been the potential selection of lysozyme-resistant clostridia. The purpose of this paper is to demonstrate that it is the conversion of spore cells into vegetative cells that is delayed by lysozyme even if spores have been derived from lysozyme-resistant vegetative cells of *C. tyrobutyricum*.

† Deceased on 7 December 1978.

MATERIALS AND METHODS

Crystalline, lyophilized egg white lysozyme with a specific activity of about 25,000 U mg^{-1} was purchased from Serva Feinbiochemica (Heidelberg, Germany). The activity of lysozyme was determined with a suspension of *Micrococcus luteus* (Serva Feinbiochemica), 1 unit catalyzing a decrease in absorbance at 450 nm of 0.001 min^{-1} at 25°C and pH 7.0. The final concentration of lysozyme in most experiments was 500 U ml of $medium^{-1}$, a concentration that does not notably interfere with the acid production of lactic starter cultures needed for the production of cheese (5).

C. tyrobutyricum strains 51, 611, and BZ15 were originally obtained from The Netherlands Dairy Research Center (NIZO, Ede, The Netherlands). Strain K1/2 was isolated in our own laboratory from cheese having a late gas defect. The clostridia were grown in glass tubes containing 10 ml of thioglycolate broth (no. 8190, Merck, Darmstadt, Germany) or in reinforced clostridial medium (RCM; Merck, no. 5411). For protection from air, the inoculated vials were sealed with 2 ml of sterile, liquefied paraffin. Enumeration of viable vegetative cells and spores was performed by the Koch pour plate count technique in reinforced clostridial agar (Merck, no. 5410). Incubation was at 37°C in an atmosphere of 90% N_2 and 10% CO_2. Spores in the dilutions were heat activated for 15 min at 75°C before addition to the medium. Although gas bubbles and cracks developed in the agar plates, they did not interfere with the counting of colonies. This method proved to be much more reproducible than the determination of viable cells by the most-probable-number procedure.

Fermentation of lactate was investigated in RCM (pH 6.1) with lactate substituting for glucose (4).

Outgrowth of spores into vegetative cells was determined in RCM (pH 5.7 to 5.8) to be 90% within 3 h of incubation at 37°C (1).

Turbidity measurements of clostridial cultures were done in an Elko III photometer (Carl Zeiss, Oberkochen, Germany) equipped with filter S59E.

RESULTS

To characterize the mechanism of inhibition of late gas formation in cheeses infected with lactate-fermenting *C. tyrobutyricum* (6), the action of lysozyme on resting and proliferating vegetative cells, as well as on spores and spore germination, was investigated.

Action of lysozyme on resting cells and spores. Figure 1 clearly demonstrates that more than 90% of viable resting cells of *C. tyrobutyricum* strains 51 and 611 were inactivated within 2 h of incubation with 500 U of lysozyme ml^{-1}. If the same experiment was conducted with spores of the same strains, no influence of lysozyme on the number of viable spores could be detected within 72 h of incubation if 1.37×10^5 (strain 51) and 0.5×10^5 (strain 611) spores per ml of medium were used.

Action of lysozyme on proliferating cells. Growth of proliferating cells of *C. tyrobutyricum* was increasingly retarded by increasing amounts of lysozyme, as shown for strain BZ15 in Fig. 2. Very similar results were obtained with strains 51, 611, and K1/2. The bacteria isolated from the lysozyme-containing vials at the end of the incubation period (8 to 11 days) proved to be resistant to lysozyme at different levels. In the case of strain 51, investigated in detail in this respect, the cells isolated from the tube containing 1,000 U of lysozyme ml^{-1} after 11 days grew as fast as the control culture without addition of lysozyme. Such resistant cells were used to produce spore suspensions to evaluate the effect of lysozyme on the outgrowth of spores into vegetative rods.

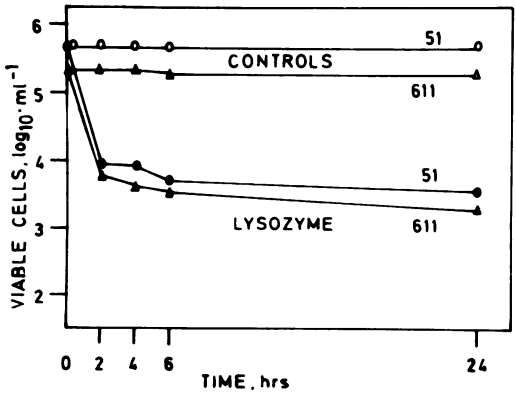

FIG. 1. *Action of lysozyme (500 U ml^{-1}) on resting vegetative cells of C. tyrobutyricum strains 51 and 611 suspended in 0.1% peptone broth. Incubation temperature was 25°C. Viable cells were enumerated by the plate count technique.*

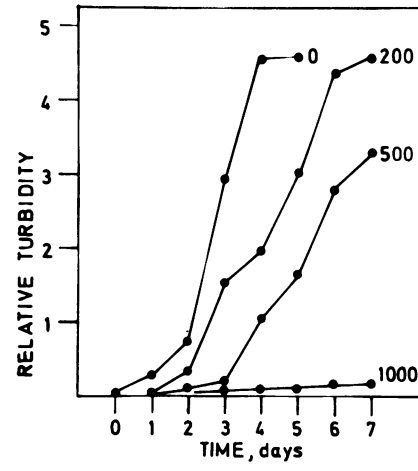

FIG. 2. *Inhibition of growth of C. tyrobutyricum BZ15 by increasing concentrations of lysozyme (0, 200, 500, and 1,000 U ml of thioglycolate medium^{-1}). The experiment was performed at 25°C. Inoculation of the lysozyme-containing medium was with 1% of a fresh culture grown for 24 h at 37°C in the same medium.*

Action of lysozyme on spore germination and conversion of spore cells into vegetative cells. No significant influence of lysozyme could be demonstrated in early events of germination, including loss of refractility and heat stability. Although 60 to 90% of all spores lost refractility within 2 h of incubation at 37°C in a suitable medium (1), depending on the strain, this effect occurred irrespective of the presence of 500 U of lysozyme ml^{-1} and irrespective of the method used to monitor this phenomenon (phase-contrast microscopy and light scattering). The same holds true for the loss of heat stability, which was also independent of lysozyme. In addition, both processes were independent of the history of the spores with regard to the lysozyme susceptibility or resistance of the mother cells.

To assess the action of lysozyme on the conversion of spore cells into proliferating vegetative cells, spores of *C. tyrobutyricum* were suspended in a germination- and growth-supporting medium (RCM or RCM-lactate) at concentrations of 4×10^5 to 2×10^6 ml^{-1}. If addition of

lysozyme inhibits overall conversion into vegetative cells, the turbidity of the growth medium should not change. If outgrowth of spores does proceed and if the formed vegetative cells are able to multiply, the developing turbidity of the medium should at least give information on the time course of the event. Figure 3 shows that clostridia started to develop 2 days after inoculation of spores into the medium. A 500-U ml^{-1} amount of lysozyme slowed down this outgrowth by 1 day, again independently of the lysozyme susceptibility or resistance of the spore mother cells.

Since lysozyme resistance is not lost by one cycle of sporulation of lysozyme-resistant vegetative cells (data not shown), the described observation of a retarded outgrowth implies that some steps in the conversion process must be sensitive to lysozyme. This explains our previous finding in practical cheese making that the late gas defect can be prevented by lysozyme even if spores from lysozyme-resistant vegetative mother cells were used for the contamination of the cheese milk (6).

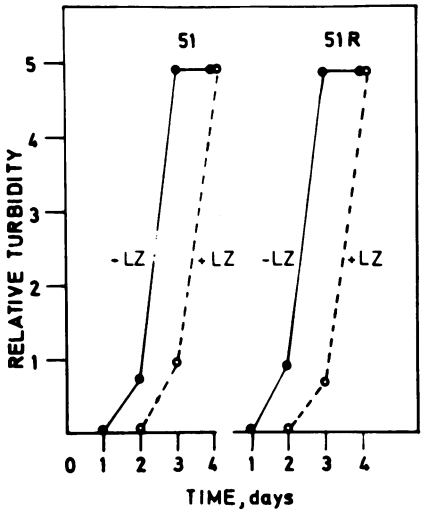

FIG. 3. *Outgrowth of C. tyrobutyricum spores into vegetative cells in RCM medium in the presence (dashed line) and absence (solid line) of 500 U of lysozyme (LZ) ml^{-1}. Data are shown for spores derived from the lysozyme-sensitive wild-type culture of strain 51 and for spores derived from lysozyme-resistant vegetative cells of strain 51, labeled 51R. The initial spore numbers were 4×10^5 ml^{-1} (strains 51 and 51R). Essentially the same curves were obtained with strains BZ15, BZ15R, 611, and 611R. Incubation was at 25°C.*

DISCUSSION

This work has clearly shown that lysozyme-resistant vegetative cells of *C. tyrobutyricum* do develop easily. Spores of *C. tyrobutyricum* are themselves resistant. The overall germination of spores into vegetative cells, however, is sensitive to lysozyme irrespective of the lysozyme susceptibility of the mother cultures of the spores. Since it is the occurrence of spores in milk that causes the late gas defect, no selective pressure can be envisaged to render the germination process resistant to lysozyme. In addition, since these spores do not originate from the cheese factory or multiply in the cheese vats, the risk of producing spores having a lysozyme-resistant germination process seems extremely unlikely. Like nitrate/nitrite, lysozyme alone is not able to completely inhibit outgrowth of spores in the test tube. In practice, the action of lysozyme is apparently aided by salt penetrating from the brine into the cheese body.

We are currently investigating the mechanism of action of lysozyme on the germination of spores of *C. tyrobutyricum*. An industrial application will depend on the availability of lysozyme at reasonable prices once the method has been tried on a technical scale.

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