Characteristics of the Inhibition of *Brochothrix thermosphacta* by *Lactobacillus brevis*  

D. L. COLLINS-THOMPSON*, D. S. WOOD† and T. J. BEVERIDGE‡  

Departments of Environmental Biology and Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1  

(Received for publication October 25, 1982)  

**ABSTRACT**  

When cultures of *Brochothrix thermosphacta* and *Lactobacillus brevis* were grown together or separated by a dialysis membrane (M.W. cut off = 3,000 daltons), the growth of *Brochothrix* was inhibited. This phenomena occurred under both aerobic and anaerobic conditions and was unaffected by the presence of catalase (412 units/ml). The antagonism appeared to be pH-mediated since it depended on glucose concentration, but low pH (4.5) alone did not directly affect the viability or salt tolerance of cells exposed to *B. thermosphacta*. Electron microscopy of thin sections of *B. thermosphacta* after 24 to 48 h of exposure to *L. brevis* revealed distinct lesions within the peripheral cell wall fabric. These were not seen in control cells of the same age or in cells exposed to 0.01 to 0.1 M acetic acid. Induction of autolysis in *B. thermosphacta* by cell wall metabolism imbalance was believed to be the cause of the growth inhibition.

There are numerous references to the phenomenon of growth antagonism among bacteria associated with food spoilage (15,17,18). Some of the most common organisms involved in antagonism are the lactic acid bacteria (9,10,19,29). In a meat environment, high numbers of these bacteria have been shown to inhibit other bacterial species (4,5,22,25). In many cases, the nature of this inhibition has not been resolved but it is linked to 'antibiotic' substances which are secreted into the growth environment by the antagonist bacteria (22,28). The existence of these substances remains in doubt, with the exception of hydrogen peroxide (3.23) and the antibiotic nisin from *Streptococcus lactis* (16). No other antibiotic substances produced by lactic acid bacteria have been clearly identified (11,14,20,27,31).

The objectives of this study were to define the conditions for growth inhibition of *B. thermosphacta* and to characterize the inhibitor produced by *L. brevis*. Knowledge about this inhibition may help to clarify some of the inconsistencies arising from the literature.

**MATERIALS AND METHODS**

**Bacterial strains**

Strains of *Lactobacillus brevis* and *Brochothrix thermosphacta* used in this study were isolated from vacuum-packed bologna and maintained as previously described by Collins-Thompson and Rodriguez Lopez (4). Growth of *L. brevis* was measured in MRS lactobacillus medium (Difco) and *B. thermosphacta* was cultured on Gardner's medium (5). Loss of salt tolerance of *B. thermosphacta* was measured on Gardner's medium containing 6% sodium chloride.

**Associated growth studies**

These studies were done at 25°C in 250-ml flasks containing 100 ml of APT broth (Difco) supplemented with 1.5% glucose (APTG) under anaerobic and aerobic conditions. Anaerobic conditions were generated using the GasPak 100 anaerobic system (BBL Microbiology Systems; 4).

Aerobic conditions were maintained by shaking the flask at 200 rev/min (924 environmental shaker, New Brunswick Scientific). A 1*10*⁶ per ml suspension of cells in 0.1% peptone water was prepared from 18-h cultures. One 10*⁶ cells/ml. Growth of the organism was monitored at 6- to 12-h time intervals for 48 h.

The catalase experiments were done under aerobic and anaerobic conditions at 25°C as described above in the presence of 412 units of catalase (Sigma Co.) per ml. Glucose studies were done using APT broth containing 0.5, 1.0 or 2.0% glucose and following counts of *L. brevis* and *B. thermosphacta* after inoculation. These studies were done under aerobic conditions at 25°C.

**Dialysis membrane studies**

Flasks containing 60 ml of APTG broth and membrane tubing (Spectrapor) with a molecular weight cut-off of approx. 3,500 daltons were sterilized. The tubing was sterilized according to manufacturer's instructions. The immersed part of the tubing was filled with 40 ml of sterile APTG broth and inoculated with 10³ cells of *L. brevis* per ml. The broth outside the tubing was inoculated with 10⁵ cells of *B. thermosphacta* per ml. Aerobic growth of the two organisms was monitored by plate counts every 6 h for 48 h.

**pH studies**

Eighteen-hour cultures of *L. brevis* grown aerobically were centrifuged for 20 min at 2880 *× g* at 4°C (Sorval RT 6000) to pellet the cells. The supernatant fluid was filter-sterilized (Nalgene 0.45 µm) and 50 ml were placed in sterile 100-ml flasks. The pH of the cell-free broth was adjusted to 4.5, 5.0, 5.5 and 6.0 with either sterile 0.1 N HCl or 0.1 N NaOH. Into each flask was inoculated approx. 10⁶ cells of *B. thermosphacta* per ml. Control flasks at pH 4.5 using fresh APTG broth were also inoculated with approx. 10⁶ cells/ml. Growth of the organism was monitored on Gardner medium and Gardner medium + 6% sodium chloride.

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*Department of Environmental Biology.*

*Department of Microbiology.*
Acetic acid studies

Acetic acid production of L. brevis and B. thermosphacta grown aerobically was determined on 24-h cultures by gas chromatographic methods using cell-free supernatant fluids. Using a flame ionization detector (Varian 1100), injection of 1.0 ml of spent broth from both cultures was made onto a Chromosorb 101 column. An internal standard of 2-pentanone (10 µl/50 L) was used. Carrier gas rates were helium (60 ml/min), hydrogen (60 ml/min), and air (250 ml/min), and column temperature was 80 to 150°C at 10°C/min.

The sensitivity of B. thermosphacta to acetic acid was determined by adding glacial acetic acid to give a final concentration of 0.01 to 0.1 M in 100 ml APTG broth. These flasks were then inoculated with 10^7 B. thermosphacta/ml, and counts of the organism were measured over a 36-h period. At a time interval of 6 h, 5 ml of broth were removed, centrifuged (10 min at 2880 x g at 4°C) and the washed cells resuspended in 0.05 M sodium cacodylate buffer (CAC) for electron microscopy studies.

Electron microscopy studies

Cells of B. thermosphacta from the dialysis membrane chamber and acetic acid studies were harvested at 6- to 12-h periods up to 48 h. Zerotime cells represented the control cultures. Each culture was suspended in 0.05 M CAC which had been previously buffered to the pH of the culture before harvesting. MgCl_2 (5 mM) was added to the buffer (CAC-Mg) to help stabilize membranes. The cells were fixed with 5% glutaraldehyde in CAC-Mg for 90 min at 22°C. They were washed in CAC-Mg and postfixed with 1% OsO_4 in CAC-Mg for 2 h at 22°C. After washing, cells were stained in aqueous 2% uranyl acetate, dehydrated through an ethanol-propylene oxide series to Epon 812, and thin-sectioned. Thin sections were stained with aqueous 2% uranyl acetate followed by lead citrate and observed using a Philips EM300 transmission electron microscope equipped with a goniometer stage.

RESULTS AND DISCUSSION

Brochothrix thermosphacta was inhibited by L. brevis under both aerobic and anaerobic conditions at 25°C (Fig. 1). Similar inhibition was observed under anaerobic conditions at 5 and 15°C (4). Although the inhibition was greater under anaerobic conditions, all further studies, with the exception of the catalase studies, were done aerobically at 25°C. These conditions were selected for ease of handling the experiments. Temperature selection was based on the optimum growth temperature for B. thermosphacta (2).

Associated growth experiments with the two organisms revealed that the inhibition of B. thermosphacta was unaffected when grown aerobically or anaerobically in the presence of catalase (Fig. 1). These results indicate that the inhibiting agent was not hydrogen peroxide. This product, often produced by lactic acid bacteria, has been shown to be inhibitory to other organisms during growth in mixed cultures (1, 3, 7, 32).

Inhibition of B. thermosphacta by L. brevis occurred in the dialysis membrane experiment at a molecular weight cut-off of approx. 3500 daltons or lower indicating that a small molecule was associated with this inhibition. Similar observations were also made by Hamden and Mikolajcik (11) with the compound acidolin from Lactobacillus acidophilus. The inhibitory molecule produced by this or-
ganism had a molecular weight of 198 daltons. Structural studies suggested that the molecule was a methyl ester. Further studies on *L. acidophilus* by Hosono et al. (14) found an inhibitory substance having a molecular weight of 3800 daltons produced by this organism. They identified this molecule as a small peptide with activity against *Escherichia coli*.

Inhibition of *B. thermosphacta* was found to be glucose dependent (Fig. 2). As the glucose concentration was raised from 0.5 to 2% in APT broth, inhibition by *L. brevis* increased dramatically. Glucose dependent inhibition by *L. brevis* has also been shown to occur against gram-negative bacteria (5). Since glucose is metabolized to primary acid products, e.g., lactic acid (21), the results show an acid-mediated inhibitor or inhibitory process. This idea was confirmed by exposing cells of *B. thermosphacta* to cell-free broth of *L. brevis* (pH 4.7) adjusted to different pH levels (Fig. 3). At pH 6.5, the spent broth supported some growth of *Brochothrix*, but as the pH dropped to 4.5, a rapid loss of viability occurred. Controls with cells of *B. thermosphacta* in fresh APT broth adjusted to pH 4.5, although showing no growth, revealed that no loss of viability occurred during a 36-h period. Acid-dependent inhibitors produced by lactic acid bacteria have been reported by other researchers. Kodama (20) showed that the activity of lactolin (*Lactobacillus plantarum*) was increased at pH 4.6. Studies on the antibacterial activity of supernatant fluids from cultures of enterococci (18) showed that inhibition against gram-positive organisms increased as the pH was lowered to 4.5.

Because of this acid sensitivity, we explored the possibility that acetic acid might be one of the components of the inhibition. *Lactobacillus brevis*, being a heterofermentative organism, produces acetic acid as a by-product of glucose metabolism. Gas chromatographic analysis of the cell-free supernatant fluids of *B. thermosphacta* and *L. brevis* showed that both organisms produced 0.036 to 0.038 M acetic acid during the 24-h growth period at 25°C. Both of these organisms at this time had reached the early stationary phase of growth. In further experiments, we found that *B. thermosphacta* was able to tolerate up to 0.1 M acetic acid at pH 4.6 for 24 h without loss in viability. Thus, under these associated growth conditions, *B. thermosphacta* would be able to tolerate the levels of acetic acid produced.

**Figure 3. Viability of *B. thermosphacta* at different pH values in APT broth supernatant fluid of *L. brevis* culture during a 36-h incubation period at 25°C.**

**Figure 4. Relationship of pH and salt sensitivity of *B. thermosphacta* on exposure to APT broth supernatant fluid of *L. brevis* during 36 h of incubation at 25°C.**

It was observed during the dialysis experiments that centrifugation of *B. thermosphacta* increased the killing effect (as measured by plate counts). This fragility of the cells was further confirmed by measuring the capacity of *Brochothrix* for growth on Gardner medium supplemented with 6% salt (Fig. 4) during pH studies with cell-free supernatant fluids of *L. brevis*. At pH 5, there were marginal differences in counts between the salt- and non-salt-containing medium. At lower pH values, the differences between the two counts became significant (i.e., 2 log₁₀). This decrease in salt tolerance was accompanied by rapid...
Figure 5 a-c. Electron micrographs of thin section of cells of B. thermosphacta showing cell wall lesions after exposure to L. brevis.
A. Control cells grown for 48 h (marker = 1000 nm; all micrographs are same magnification). B. Cells exposed to products of L. brevis for 24 h. (↑ arrows pointing to holes developing in cell wall fabric). C. Cells exposed for 48 h to products from L. brevis (↓ arrows pointing to remnants of cell wall periphery).
loss in viability. This observation, along with a decrease in optical density, suggested changes either in the cell wall or membrane structure.

Electron microscopy of thin sections of cells of *B. thermosphacta* exposed to *L. brevis* in the dialysis membrane studies confirmed this hypothesis (Fig. 5A-C). Control cells of *B. thermosphacta* grown for the same length of time as those exposed to products from *L. brevis* revealed very few lysed cells. These cells were actively dividing and possessed intact cell walls which were typical of the gram-positive variety (Fig. 5A). The 24-h-exposed cultures contained more lysed cells, and the walls of intact cells appeared to possess lesions along their periphery (Fig. 5B). These lesions were very apparent in the 48-h-old walls (Fig. 5C). In fact, the thickness of the wall fabric had been reduced by approximately 50%. The 48-h cultures contained many lysed cells and associated cellular debris. Electron microscopy of cells of *B. thermosphacta* exposed to 0.01 to 0.1 M acetic acid for a 36-h period showed no loss in cell wall thickness nor any evidence of lesions.

Zones of inhibition between *L. brevis* and *B. thermosphacta* using the disc assay method (11, 26) were not observed. Some inhibition of *B. thermosphacta* was observed when parallel streaking of the two cultures (1 cm apart) was done on APT agar plates.

The lesions in the walls of 24- to 48-h cultures seen by electron microscopy suggest that the wall is undergoing hydrolytic breakdown. These regions of hydrolysis were not localized to the sidewall, polar cap or septal regions, but were continuous around the cell periphery. Initially, we suspected that the hydrolytic effect was caused by a low molecular weight antibiotic. Tomasz et al. (30), in their report on an autolytic defective mutant, *Streptococcus* (Diplococcus) pneumoniae, suggested that autolytic activity was a prerequisite for penicillin lysis.

The simplest explanation involves autolytic enzymes. Since all known autolysins have greater molecular weights than the cut-off value of the dialysis membrane used in this study, we can exclude exogenous hydrolytic enzymes derived from the *L. brevis* culture. It is possible that *L. brevis* excretes a low molecular weight substance which activates the autolytic mechanism(s) in *B. thermosphacta* walls. This activation is more pronounced at low pH and would surpass the ability of the microorganisms to incorporate the cross-link new wall polymers to mend injured walls. If this is the case, then the wall would become fragile and the bacterium more sensitive to salt.

A similar effect has been demonstrated with *L. acidophilus*. This bacterium was shown to undergo wall autolysis (maximum activity at pH 4.5) (6). *Brochothrix thermosphacta* is considered to be an intermediate lactic acid bacteria and could possess similar autolytic mechanisms.

At present, we do not know the identity of the compound from *L. brevis*. Work with gram-positive bacteria has suggested that membrane lipoteichoic acids and wall teichoic or teichuronic acids are important for the control of autolytic activity (12, 13, 24). In the lipoteichoic acid situation, small molecules, such as ethanolamine (M.W. = 61 daltons) or choline (M.W. = 121 daltons), are capable of stringent control of the autolytic mechanism (12, 13). Although much more work is necessary to define the process involved with the *B. thermosphacta* and *L. brevis* interaction, it is tempting to speculate an analogous system. We believe that the normal equilibrium between wall hydrolysis and wall biosynthesis in *B. thermosphacta* is disturbed by an active, low molecular weight compound that is excreted into the milieu by *L. brevis*. This compound, in concert with low pH and interacting with cells of *B. thermosphacta*, would activate the autolytic process causing lysis.

**ACKNOWLEDGMENTS**

This work was supported in part by grants from the Natural Sciences and Engineering Research Council of Canada to D. L. Collins-Thompson and T. J. Beveridge, and from the Ontario Ministry of Agriculture and Food to D. L. Collins-Thompson. We also acknowledge technical support from Michelle Azan, Bill Krusky and Trims Linda.

**REFERENCES**

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Collins-Thompson et al., cont.’s from p. 407


