Inhibition, Injury, and Inactivation of Four Psychrotrophic Foodborne Bacteria by the Preservatives Methyl \(\rho\)-Hydroxybenzoate and Potassium Sorbate

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ABSTRACT

The minimum inhibitory concentrations (MICs) of methyl \(\rho\)-hydroxybenzoate (methyl paraben) and potassium sorbate for four psychrotrophic bacteria were compared at pH 5 and 6 and at 5 and 30°C. The bacteria tested were Listeria monocytogenes, Pseudomonas putida, Yersinia enterocolitica, and Aeromonas hydrophila. L. monocytogenes was generally the most resistant and A. hydrophila the least resistant to the preservatives. The differences between the bacteria were substantial. The MICs of the two preservatives were similar at pH 5, but at pH 6 the MICs of paraben were well below those of sorbate, except in the case of A. hydrophila. The MICs at 5°C were much lower than those observed at 30°C for all of the bacteria except P. putida. All four bacteria were inhibited by 1000 mg methyl paraben per L at 5°C. Exposure of the bacteria to concentrations of preservative that permitted growth at 30°C did not lead to adaptation to the preservative. The death rates of the bacteria in media containing 1000 mg methyl paraben per L varied over a wide range. At 5°C, a 3 log₁₀ decrease in viable counts of L. monocytogenes and A. hydrophila took >4 months and a few days, respectively. Injury of L. monocytogenes, Y. enterocolitica, and A. hydrophila was detected under these conditions. Repair of the injury was demonstrated, with up to 24 h required for complete recovery. The type of buffer in which the test medium was prepared affected the preservative MICs and rate of injury of L. monocytogenes.

The growing market for chilled convenience foods has increased the importance of psychrotrophic (cold-tolerant) bacteria for the food industry (14,22). Pathogens capable of growth at temperatures encountered commonly in chilled foods include Listeria monocytogenes, Yersinia enterocolitica, Aeromonas hydrophila, and nonproteolytic Clostridium botulinum (8,15). Psychrotrophic members of Pseudomonas and other genera frequently spoil refrigerated foods (11,25,29).

Chemical preservatives may help to control psychrotrophic microorganisms in chilled foods. Potassium sorbate has been used extensively as a preservative, at concentrations up to approximately 4000 mg/kg (3000 mg/kg calculated as the acid; 26). Esters of \(\rho\)-hydroxybenzoic acid (parabens) have been permitted in foods in European countries since the 1930s and, more recently, in the United States. Sorbate and parabens are used in fruit juices and juice products, beverages, bread and other flour products, cheeses and other dairy products, jams, preserves, wines, and other foods (5,26).

Parabens have potential advantages over sorbate and other relatively pH-dependent antimicrobials. Organic acids are most active against microorganisms when their undissociated form is present (24). A large proportion of paraben is in the undissociated form at the pH values of most foods because its pKa is 8.5. Therefore, parabens are effective over a wide pH range, pH 4–8 (1). The pKa of sorbic acid is 4.74, so its antimicrobial activity diminishes as the pH value rises towards neutrality. The behavior of psychrotrophic bacteria in the presence of these preservatives, particularly the parabens, has not been studied intensively.

This study examined the responses of L. monocytogenes, Y. enterocolitica, A. hydrophila, and Pseudomonas putida to potassium sorbate and methyl paraben. The minimum inhibitory concentrations (MICs) of potassium sorbate and methyl paraben for these bacteria were determined at temperatures simulating chilled storage (5°C) and abuse of foods (30°C). The effects of changes in pH value on the MICs were assessed. Experiments were performed to determine whether the MIC of preservative increases after the bacteria are exposed to concentrations of preservative that permit growth. This phenomenon, referred to as adaptation, has been observed with some other microorganisms (7,30). The patterns of injury and inactivation of the four bacteria in concentrations of methyl paraben that do not permit growth were compared. Subsequent resuscitation of injured bacteria was also examined.

Data on the responses of pathogens to these chemicals are essential to the safe use of preservatives by the food industry. If a preservative is not sufficiently inhibitory for pathogens, its use may create a hazard by favoring the...
survival and/or growth of a pathogen over spoilage microorganisms in a product.

Preservative-induced injury is particularly significant in the detection of microorganisms in foods. Injury caused by an environmental stress is usually manifested in the laboratory as inability to grow in selective media that support the growth of uninjured cells (4). Thus, injured cells may not be detected if selective media are used without prior resuscitation. A hazard exists if injured bacteria are not detected, because repair and growth of injured cells may occur in food. There has been substantial research on the injury of microorganisms by food processing operations (4,9,12,18). However, most studies have involved physical treatments such as heating, freezing, chilling, irradiation, and drying.

MATERIALS AND METHODS

Test bacteria and cultural conditions

The bacteria used were L. monocytogenes Scott A, Y. enterocolitica 03 (both from New South Wales Dairy Corporation, Sydney), A. hydrophila UQM 2768 (from the Department of Microbiology, University of Queensland), and P. putida P14 (from Australian Cooperative Foods Pty Ltd, Sydney). Cultures used in all experiments were grown in brain heart infusion broth (BHI; Oxoid, Basingstoke, England) for 18-24 h at 30°C, except for L. monocytogenes which was grown at 37°C. Growth curves showed that cultures prepared in this way were in stationary phase.

Unless stated otherwise, viable counts were performed by the surface spread technique on brain heart infusion agar (BHIA; Oxoid) plates with peptone solution (1 g/L) as the diluent. Plates were incubated at 30°C, with the exception of L. monocytogenes plates, which were incubated at 37°C.

Media

BHI used in MIC determinations, adaptation experiments, and injury/inactivation experiments was prepared according to the manufacturer's instructions, except that water was replaced by citric acid/phosphate buffer or phosphate buffer, pH 5 or 6 (15). Minor pH adjustments were made to the complete medium with hydrochloric acid to give a final pH value of 5 or 6. BHI containing citric acid/phosphate buffer was used for experiments with Y. enterocolitica and P. putida. Phosphate-buffered medium was used in experiments with A. hydrophila and L. monocytogenes, unless stated otherwise, because these bacteria did not grow well in citric acid/phosphate buffer under some conditions.

Sterile solutions (10% w/v) of potassium sorbate and methyl paraben were prepared in distilled deionised water and 50% (v/v) ethanol, respectively. These solutions were added aseptically to sterile buffered BHI medium to give the required preservative concentrations. The pH of the media after addition of the preservative solutions was pH 5 or 6 ± 0.05.

Determination of MIC of preservative

A culture was diluted to the desired concentration in buffered BHI at a pH value appropriate to the experiment. A series of screw-cap tubes (16-mm diameter) containing buffered BHI broths with a range of preservative concentrations was prepared. Each tube contained 5 ml of medium and was inoculated with 0.5 ml of the suspension of bacteria. The preservative concentrations in the tubes after inoculation increased in steps of 50 mg/L up to 600 mg/L, then steps of 100 mg/L up to 2000 mg/L, and steps of 200 mg/L above 2000 mg/L. The initial count of bacteria in each inoculated tube was approximately 10⁵ CFU/ml. The tubes were incubated and examined periodically for visible growth. The MIC was the lowest concentration which prevented visible growth after 10 d at 30°C or 3 months at 5°C. Preliminary experiments showed that growth was rarely detected after longer incubation times.

Adaptation

One hundred μl of culture were inoculated into 10 ml of buffered BHI (pH 6) containing preservative, then inoculated for 18 to 24 h at 30°C. The MIC of the preservative for the cultures grown in the presence of preservative was compared with that for control cultures tested in parallel.

Inactivation and injury of bacteria by methyl paraben

Bacteria were inoculated into buffered BHI (pH 6) at a concentration of approximately 10⁴ CFU/ml using the method described above. The buffered BHI contained 1000 mg methyl paraben per L after inoculation. This concentration, which is within the range of concentrations commonly used in foods, was sufficient to prevent growth of all of the test bacteria at both 5 and 30°C, except for L. monocytogenes at 30°C. The inoculated tubes were incubated at 5 or 30°C and the viable count of each suspension was determined at appropriate intervals. Viable counts were performed in parallel on BHIA and a selective medium to detect total viable bacteria (uninjured plus injured cells) and uninjured bacteria only, respectively.

Selective media used were: tryptose phosphate agar containing 65 g NaCl per L (TPSA; 10) for L. monocytogenes, violet red bile agar (VRBA; Oxoid) for A. hydrophila and Y. enterocolitica, and Pseudomonas Agar with C-F-C supplement (PSA; Oxoid) for P. putida. BHIA with added NaCl was also used as a selective medium for A. hydrophila, P. putida, and Y. enterocolitica. The concentrations of NaCl used for these bacteria were 20, 35, and 45 g/L, respectively. The concentrations of NaCl to be used were determined by performing viable counts of stationary phase cultures on BHIA containing a range of salt concentrations. The highest concentration that did not reduce the viable count was used.

Where low viable counts were expected, inhibitory effects of preservative in media used for enumeration were avoided by using a membrane filtration technique. Iso-grid hydrophobic grid membrane filters and filtration units without prefilters (QA Laboratories) were used. The test sample was suspended in 5 ml of peptone diluent on a membrane in a filtration unit. A vacuum was applied to draw the liquid through the filter, then the membrane was removed and placed on the agar medium. After incubation, counts were calculated as described by Brodsky et al. (3). Membrane filtration counts and surface plate counts gave equivalent results under the conditions used here. All experiments were performed at least twice to ensure that the results were reproducible.

Resuscitation

Repair of injury was observed as follows. Samples of a suspension of bacteria injured by exposure to methyl paraben were drawn through a series of Iso-grid membranes as described above. The membranes were placed on BHIA and incubated at 30 or 37°C (L. monocytogenes only). A membrane was transferred from BHIA onto a selective medium at each of a series of sampling times, reincubated, then colonies were counted. One membrane was not transferred to selective medium. The percentage of uninjured cells at each time was calculated as follows:

$$\text{% uninjured cells} = \frac{\text{count from selective medium}}{\text{count from BHIA}} \times 100$$
Minimum inhibitory concentrations

In general, L. monocytogenes was the most resistant to the preservatives and A. hydrophila the least resistant of the bacteria tested (Tables 1, 2). At 30°C, the MICs of the two preservatives for A. hydrophila were similar at pH 6. The MICs of paraben were substantially below those of sorbate for the other species at this pH (Table 1). A reduction in the pH of the test medium to pH 5 reduced all of the MICs and substantially diminished the differences between the MICs of the two preservatives and between organisms that were observed at pH 6. The influence of pH on the antimicrobial activity of sorbate was reflected in the dramatic reduction of MICs when the pH was decreased to 5. The reduction in MICs of paraben as the pH was decreased was small in comparison with that observed with sorbate. Experiments with L. monocytogenes showed that the buffer used in the medium influences the MIC, with higher MICs obtained in the phosphate buffer.

TABLE 1. MICs of methyl paraben and potassium sorbate (mg/L) at 30°C. The time (d) to detect growth in the highest concentration of preservative that permitted growth is in parentheses.

<table>
<thead>
<tr>
<th>pH 5</th>
<th>pH 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer Paraben Sorbate</td>
<td>Paraben Sorbate</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>C 300 (5) 400 (10)</td>
</tr>
<tr>
<td>P 700 (7) 600 (8)</td>
<td>1600 (6) &gt;5000 (6)</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>C 250 (4) 200 (6)</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td>P 200 (6) 50 (1)</td>
</tr>
<tr>
<td>P. putida</td>
<td>C 300 (10) 250 (3)</td>
</tr>
</tbody>
</table>

* Buffer in which the test medium was prepared.

P. putida was much more resistant to sorbate and slightly more resistant to paraben at the lower temperature.

The MICs of paraben were very reproducible in 3-6 replicate determinations performed on different days. The MICs of sorbate varied over about a twofold range for each organism, despite careful attention to detail in the preparation of media and suspensions of bacteria, incubation conditions, and other factors. The tables show the maximum values obtained for sorbate.

The time that elapsed before growth was observed increased in the presence of preservative. For example, growth of all strains was observed after 1 d in control tubes at pH 6 and 30°C, whereas in tubes containing preservative, particularly at concentrations close to the MIC, growth was often not observed until 7 d incubation.

Adaptation

Overnight growth at 30°C and pH 6 in subinhibitory concentrations of paraben or sorbate did not increase the resistance of the bacteria to the preservatives (Table 3). In some cases, growth was detected a few days earlier in tubes inoculated with the treated cultures than in the control tubes, suggesting that some form of habituation might have occurred.

TABLE 3. Effect of growth in the presence of a preservative at pH 6 on the subsequent resistance to that preservative. MICs were determined at pH 6 and 30°C.

<table>
<thead>
<tr>
<th>Growth medium for the inoculum used in MIC determinations</th>
<th>Sorbate</th>
<th>Paraben</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. monocytogenes BHI</td>
<td>&gt;6000</td>
<td>1200</td>
</tr>
<tr>
<td>BHI + 200 mg paraben/L</td>
<td>1200</td>
<td></td>
</tr>
<tr>
<td>Y. enterocolitica BHI</td>
<td>1100</td>
<td>350</td>
</tr>
<tr>
<td>BHI + 1000 mg sorbate/L</td>
<td>1100</td>
<td></td>
</tr>
<tr>
<td>BHI + 200 mg paraben/L</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>A. hydrophila BHI</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td>BHI + 200 mg sorbate/L</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td>BHI + 200 mg paraben/L</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>P. putida BHI</td>
<td>2400</td>
<td>400</td>
</tr>
<tr>
<td>BHI + 1000 mg sorbate/L</td>
<td>2800</td>
<td></td>
</tr>
<tr>
<td>BHI + 200 mg paraben/L</td>
<td>400</td>
<td></td>
</tr>
</tbody>
</table>

Inactivation and injury of bacteria by methyl paraben

A variety of responses was observed when the bacteria were stored at 5 or 30°C in broth containing methyl paraben (1000 mg/L, pH 6). The ability of the test strains to survive under these conditions differed markedly, particularly at 5°C, and was ranked in the same order as their resistance to the preservative (Fig. 1-4). Inactivation of all organisms except L. monocytogenes was much more rapid at 30°C than at 5°C. L. monocytogenes grew at 30°C under these conditions.

L. monocytogenes survived well at 5°C in the presence of paraben. The count on nonselective medium declined slowly to about 1% of the initial value after 4 months in both citrate and phosphate-buffered BHI (Fig. 1). Under the same conditions a similar decline in counts took 1-2 weeks for P. putida (Fig. 2) and Y. enterocolitica (Fig. 3), and a few days for A. hydrophila (Fig. 4). The rate at which injury of L. monocytogenes became apparent at 5°C varied with the buffer used in the medium.
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Figure 1. Inactivation and injury of L. monocytogenes at 5°C in BHI (pH 6) containing 1000 mg methyl paraben per L at 5°C (●) and 30°C (■). PSA was used to enumerate uninjured bacteria. Closed symbols, count on BHIA (total viable bacteria); open symbols, count on selective medium (uninjured bacteria). Arrows indicate that the count was less than the value shown by the data point immediately above the arrow; the value shown is the limit of detection of the test.

Injury occurred more rapidly and affected a greater proportion of cells in the citrate-buffered medium (Fig. 1). After 2 months, >99.9 and 86% of CFUs of L. monocytogenes were injured in citrate and phosphate-buffered media, respectively, in the experiments shown in Fig. 1. Approximately 3-6% of L. monocytogenes remained uninjured after almost 4 months in the phosphate medium.

Injury of Y. enterocolitica and A. hydrophila was demonstrated using salt-based and bile-based selective agars (Fig. 3, 4). The observed degree of injury of Y. enterocolitica varied with the selective medium used (Fig. 3). Injury of A. hydrophila did not vary markedly with the selective medium. Injury of P. putida was not detected using either BHIA containing up to 35 g NaCl per L or PSA (Fig. 2). Fig. 2 and 4 show data for only one selective medium.

Resuscitation

Repair of injury caused by storage in broth containing paraben was observed (Fig. 5). A. hydrophila, which was injured and inactivated most rapidly, also repaired most rapidly. About 70% of A. hydrophila were uninjured after 4 h of resuscitation, increasing to 86% after 8 h and 100% after 24 h.

Only 30 to 40% of L. monocytogenes were uninjured after 4 h of resuscitation, with no substantial increase in the number of uninjured cells in the following 4 h. A secondary increase in the proportion of cells repaired was apparent between 8 and 24 h. Resuscitation of L. monocytogenes injured in both citrate-buffered and phosphate-buffered BHI followed the same pattern.

DISCUSSION

The psychrotrophic bacteria studied show substantial differences in their resistance to preservatives. At pH 6 the lowest MIC of sorbate observed (for A. hydrophila) was at least tenfold lower than the highest (for L. monocytogenes at 30°C and P. putida at 5°C). The difference between the highest and lowest MICs of paraben was smaller, but still considerable under most conditions. These differences suggest that preservatives will change substantially the composition of the microflora of chilled foods in which they are used, by being more inimical for some bacteria than for others. Some pathogens might be among the more preservative-tolerant bacteria present. The data show that L. monocytogenes, the psychrotrophic pathogen of most concern to much of the food industry at present, tolerates these preservatives relatively well. Thus, it is important to take into account both spoilage and pathogenic bacteria when using preservatives. Products must be formulated to inhibit the most resistant organisms of concern.

The results show that interactions between bacteria, foods, and preservatives are complex. Inhibition of the psychrotrophs by sorbate and paraben was affected by...
temperature and pH, but changes in the conditions under which the MICs were determined did not affect all of the bacteria equally. Incubation at 5°C rather than 30°C decreased the MICs of the preservatives for the pathogens, but not for P. putida. This phenomenon is unusual since low temperature, low pH, and preservatives generally have additive inhibitory effects (23). The MIC of sorbate was very much higher than the MIC of paraben at pH 6 for all of the bacteria except A. hydrophila. Thus, extrapolation from one set of conditions to another or from one organism to another is not valid when the inhibitory effects of preservatives are being considered.

Methyl paraben has a greater potential for use than sorbate in foods with relatively high pH values. Methyl paraben inhibited the test strains at much lower concentrations than potassium sorbate at pH 6, but this difference generally disappeared at pH 5. Growth of all the bacteria was inhibited at pH 6 and 5°C by paraben concentrations below 1000 mg/kg, the level classified as GRAS in the United States (5).

The MICs observed in this study are generally consistent with those reported by other workers (2,16), although differences in experimental conditions sometimes make comparisons difficult. Two strains of P. fluorescens grew at 24°C in trypticase soy broth with 2000 mg sorbate per L at pH 6, but not in 500 mg sorbate per L at pH 5.5 (21). Growth of two strains of Y. enterocolitica was inhibited by 1000 mg sorbate per L BHI at pH 5.5 and 25°C and 250 mg sorbate per L at 3°C (28). However, the experiments at 3°C were continued for only 3 d. The current study showed clearly that short incubation periods (a few days) at low temperatures do not yield realistic MICs, because growth may not be apparent for some weeks. Chilled foods may be stored for several weeks.

Growth of L. monocytogenes has been observed by other workers at 13, 21, and 35°C at pH 5.6 in sorbate concentrations up to and including 3000 mg/L (6). At 4°C and pH 5.6 very slight growth was observed in sorbate concentrations of 1000 mg/L but not in 1500 mg/L. At pH 5, growth was not observed at 4°C, but 1500 and 2000 mg sorbate per L were required for inhibition of L. monocytogenes at 21 and 35°C, respectively, substantially higher concentrations than were required in the current study. This difference might be related to strain-to-strain variation or to differences between media and buffering systems, which can have important effects on the growth of L. monocytogenes (27). The stage in the growth cycle at which the test organisms are exposed to preservative might also influence the results. Cultures in stationary phase were used in the experiments reported here.

The rate at which the bacteria were inactivated in BHI containing 1000 mg paraben per L was inversely related to their preservative resistance. The ability of L. monocytogenes to survive for months in inhibitory concentrations of paraben at low temperatures is potentially hazardous.

The differences between the bacteria in inactivation rates, degrees of injury, and time required for resuscitation suggest that there may be differences in the sites of damage and/or degree of damage of the same site. For example, A.
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The apparent two-stage recovery of *L. monocytogenes*, *Y. enterocolitica*, and possibly *A. hydrophila*, suggests that two types of injury might be occurring. Cells which are repaired in the first stage have probably experienced only one type of injury, which can be repaired rapidly. Cells that require longer periods for recovery may have experienced more severe injury, probably to several cellular components. The pause in the recovery process before the second stage of repair suggests that cells might have sustained damage at multiple sites and must recover from one type of damage before a second stage of repair can occur.

Organic acids such as citrate have been shown to potentiate the antimicrobial action of sorbate (20). Paraben-induced injury of *L. monocytogenes* at 5°C occurred more rapidly in the citrate medium than when the phosphate medium was used. However, the same pattern of resuscitation was observed, regardless of the buffer in which injury occurred. This suggests that damage is occurring at the same site(s) and that it was the level of injury that varied in the two buffers.

Specific resuscitation requirements need to be determined for use during the detection of foodborne bacteria injured by preservatives. One to 4 h are often allowed for repair of injured vegetative cells, depending on the type of stress (19). The resuscitation experiments described here show that longer periods may be required for repair of injury caused by preservatives.

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REFERENCES