Bibersteinia trehalosi Inhibits the Growth of Mannheimia haemolytica by a Proximity-Dependent Mechanism

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Mannheimia (Pasteurella) haemolytica is the only pathogen that consistently causes severe bronchopneumonia and rapid death of bighorn sheep (BHS; Ovis canadensis) under experimental conditions. Paradoxically, Bibersteinia (Pasteurella) trehalosi and Pasteurella multocida have been isolated from BHS pneumatic lungs much more frequently than M. haemolytica. These observations suggest that there may be an interaction between these bacteria, and we hypothesized that B. trehalosi overgrows or otherwise inhibits the growth of M. haemolytica. Growth curves (monoculture) demonstrated that B. trehalosi has a shorter doubling time (~10 min versus ~27 min) and consistently achieves 3-log higher cell density (CFU/ml) compared to M. haemolytica. During coculture M. haemolytica growth was inhibited when B. trehalosi entered stationary phase (6 h) resulting in a final cell density for M. haemolytica that was 6 to 9 logs lower than expected with growth in the absence of B. trehalosi. Coculture supernatant failed to inhibit M. haemolytica growth on agar or in broth, indicating no obvious involvement of lytic phages, bacteriocins, or quorum-sensing systems. This observation was confirmed by limited growth inhibition of M. haemolytica when both pathogens were cultured in the same media but separated by a filter (0.4-µm pore size) that limited contact between the two bacterial populations. There was significant growth inhibition of M. haemolytica when the populations were separated by membranes with a pore size of 8 µm that allowed free contact. These observations demonstrate that B. trehalosi can both outgrow and inhibit M. haemolytica growth with the latter related to a proximity- or contact-dependent mechanism.

The bighorn sheep (BHS; Ovis canadensis) population in North America has declined from an estimated two million at the beginning of the 19th century to fewer than 70,000 today (7, 30). The decline of BHS populations is presumably due to loss of habitat, competition for forage with domestic livestock, predation, and disease (9, 19). The most important disease that has limited the growth of BHS populations is pneumonia (13, 14, 19, 31). Bacteria associated with BHS pneumonia are members of the genera Mannheimia and Pasteurella, particularly, the species Mannheimia (Pasteurella) haemolytica, Bibersteinia (Pasteurella) trehalosi, and Pasteurella multocida (6–9, 15, 20, 25, 31). Several independent studies have revealed that M. haemolytica is the major cause of BHS pneumonia. In fact, M. haemolytica is the only pathogen that has been shown to consistently cause severe bronchopneumonia and rapid death of BHS under experimental conditions (10, 14, 23). B. trehalosi has been isolated more often than M. haemolytica from the upper respiratory tract of healthy BHS (10, 12–14, 26, 31). Large numbers of B. trehalosi have also been isolated from the pneumatic lungs of BHS experimentally inoculated with M. haemolytica alone (10). Furthermore, our recent studies with M. haemolytica wild type and leukotoxin deletion mutants in BHS have revealed that the leukotoxin deletion mutant does not cause the death of BHS but instead induces only mild lung lesions, confirming the finding in cattle that leukotoxin is the most important virulence factor of M. haemolytica (10, 24, 29). Our recently concluded BHS inoculation study revealed that only leukotoxin producing strains of B. trehalosi can cause pneumonia, indicating that leukotoxin is the most important virulence determinant in B. trehalosi as well. More than 85% of the B. trehalosi isolates obtained from BHS, however, do not produce leukotoxin (28, 32). Therefore, this observation, together with the results from the animal experiments, indicates that B. trehalosi is unlikely to be the major cause of pneumonia outbreaks in BHS.

These observations prompted us to hypothesize that B. trehalosi outgrows or otherwise inhibits the growth of M. haemolytica. The objectives of the present study were to (i) characterize in vitro growth kinetics of M. haemolytica and B. trehalosi; (ii) develop M. haemolytica-specific and B. trehalosi-specific PCR assays to detect either species in mixed cultures; and (iii) determine whether B. trehalosi inhibits the growth of M. haemolytica in vitro and, if it does, characterize the mechanism of inhibition.

MATERIALS AND METHODS

Bacterial strains and culture conditions. M. haemolytica and B. trehalosi cultures were maintained as frozen stocks (~80°C) in brain heart infusion broth (BHI) with 10% glycerol. From the stocks, M. haemolytica serotype A1 (80010807N [21]) and other BHS isolates, as well as B. trehalosi serotype T20 (ATCC 33374) and other BHS isolates, were individually cultivated in BHI agar supplemented with 5% defibrinated sheep blood (Remel, Lenexa, KS) and 37°C overnight incubation. A loopful of bacteria was transferred into each one of several tubes containing 10 ml of BHI broth and cultured overnight at 37°C with constant shaking (200 rpm). The chromosomally encoded ampicillin-resistant M.

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haemolytica strain developed by Murphy et al. (21) was cultured in BHI containing 10 μg of ampicillin/ml. We developed a rifampin-resistant B. trehalosi strain by culturing the wild-type B. trehalosi on BHI agar plates containing 10 μg of rifampin/ml overnight. The resistant clones were isolated and maintained in medium with rifampin. Antibiotic-sensitive B. trehalosi and M. haemolytica, as well as ampicillin-resistant (Amy) M. haemolytica and rifampin-resistant (Rif) B. trehalosi were used in subsequent studies.

Isolation of bacteria from pulmonary BHS lungs. Dacron swabs taken from the lungs were inoculated onto Columbia blood agar (Becton Dickinson, Sparks, MD) supplemented with 5% (vol/vol) sheep blood and onto a Pasteurellaceae selective agar medium (16, 17). Each plate was streaked for colony isolation. The plates were incubated at 37°C in 10% CO2 and inspected at 24 and 48 h. All representative colonies were picked and grown in wedges on Columbia blood agar. Isolates were determined to be M. haemolytica or B. trehalosi if they were Gram stain negative; pleomorphic; MacConkey, urea, and indole negative; oxidase, nitrate, glucose, and sucrose positive; and xylene or trehalose positive. Each isolate was biovariant-based on its ability to ferment various sugars in addition to other biochemical tests (a total of 21 phenotypic characteristics) according to the protocol of Jaworski et al. (16). P. multocida isolates fit the above criteria except that they were indole positive. P. multocida was further characterized according to a previously described protocol (5).

Bacterial growth curves. Overnight cultures of B. trehalosi and M. haemolytica were diluted and inoculated (–10 CFU) separately into two culture tubes, each containing 10 ml of BHI broth, followed by incubation (37°C, 200 rpm). At multiple time points up to 24 h, the optical density at 600 nm of the cultures was recorded, and a small aliquot of the culture was taken for CFU counts. Samples collected at each time point were serially diluted with BHI broth, placed on BHI agar plates, and incubated at 37°C overnight. After overnight growth, bacterial colonies were counted and plotted against time.

Plate assay. Supernatant fluid was collected from single cultures of B. trehalosi or M. haemolytica or cocultures of B. trehalosi and M. haemolytica at different time points (1, 6, 12, and 24 h) and filter-sterilized (Acrodisc 0.2- or 0.45-μm-pore-size Supor membrane syringe filters; Pall filters (Acrodisc 0.2- or 0.45-μm pore size)) during filtration, culture supernatants were filtered through low protein binding adsorption of bacteriophage (if any), after which 3 ml of BHI soft molten agar was added. The 5 ml of filter-sterilized culture supernatant fluid (5 ml) was mixed with equal volumes of BHI agar plates, followed by colony PCR (described below). In the same coculture experiments, antibiotic sensitive bacteria were replaced with Amy M. haemolytica and Rif B. trehalosi strains. Serially diluted bacteria from the individual cultures and cocultures collected at different time points were plated either on ampicillin plates (for M. haemolytica) or rifampin plates (for B. trehalosi) and cultured at 37°C overnight. Colonies in each of the plates were counted and expressed as CFU/ml.

Multiplex PCR assay. To differentiate M. haemolytica from B. trehalosi, O-sialoglycoprotein endopeptidase (gsp; GenBank accession numbers AY702251 and AY702252) sequences were aligned by using CLUSTAL W program (http://www.ebi.ac.uk/Tools/clustalw). The regions exhibiting the least degree of sequence identity were selected, and the following species-specific primers were designed for multiplex PCR assay: M. haemolytica-specific gsp forward primer Mhgfp (5'‐AGA GCC CAA TCT GCA AAC TCT-3') and reverse primer Mhgrp (5’‐GTT CGT ATT GTC CAC CGC‐3'); B. trehalosi-specific sod forward primer BtsodF (5’‐GCC TGC GGA CAA CAC TGT TG3‐3') and reverse primer BtsodR (5’‐TTT CAA CAG AAC GCT GGT CAC GAA T‐3'). Multiplex PCR was performed using a total volume of 50 μl with GoTaq PCR SuperMix (Promega, Madison, WI), with 0.2 μM concentrations of each primer and 2 μl of bacterial culture (colony or broth). The PCR cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 40 s, and a final elongation at 72°C for 5 min. The PCR products were visualized (gsp, 267 bp; sodA, 144 bp) after electrophoresis through 1% agarose gels run at 7.0 V/cm and stained with ethidium bromide. The specificity of the multiplex PCR primers for M. haemolytica and B. trehalosi was evaluated by using multiple field and reference strains. At least 30 individual colonies at each time point were assessed by the multiplex PCR.

Proximity-dependent inhibition assay. To determine whether B. trehalosi-mediated growth inhibition of M. haemolytica was due to contact-dependent inhibition, we used cell culture inserts with two different pore sizes in the competition assay. Generally, bacteria do not pass through 0.4-μm pores, but they can readily pass through 8.0-μm pores, whereas macromolecules such as bacteriocin and other molecules can pass through 0.4-μm pores by simple diffusion. Therefore, we used 0.4- and 8.0-μm-pore-size polycarbonate terephthalate (PET) track-etched membrane cell culture inserts (BD Falcon; BD Biosciences, Franklin Lakes, NJ) to further characterize the observed growth inhibition. B. trehalosi inserts in six-well cell culture plates create upper and lower chambers. Approximately 10^5 CFU of Rif B. trehalosi in 10 μl of BHI were added into the upper chambers containing 2.5 ml of antibiotic-free BHI, and ~10^5 CFU of Amy M. haemolytica (at a 10:1 inhibitor/target ratio) in 10 μl of BHI were added into the lower chambers containing the same volume of antibiotic-free BHI. The culture plates were covered with the lids and wrapped with Parafilm to minimize evaporation of the culture medium. These plates were incubated at 37°C with constant shaking at 100 rpm. Samples from both upper and lower chambers were collected at 24 h postincubation and serially diluted with BHI broth and placed on BHI agar plates containing either ampicillin or rifampin. As controls, Rif B. trehalosi and Amy M. haemolytica were individually cultured in six-well culture plates with the same culture volume, and CFU assays were performed.

Statistical analysis. B. trehalosi and M. haemolytica numbers at different time points were expressed as mean CFU/ml with their corresponding standard deviations. The data were statistically analyzed by the Student t test, and P values were determined by using NCSS 2004 (Number Cruncher Statistical System, Kaysville, UT). The term “significant” indicates P < 0.05 with corrections for experimental error made using Bonferroni intervals.

RESULTS AND DISCUSSION

Prevalence of B. trehalosi, M. haemolytica, and P. multocida in pulmonary BHS lungs. Bacteria associated with BHS pneumonia are members of the genera Mannheimia and Pasteurella.
TABLE 1. Bacteria isolated from pneumonic lungs of BHS

<table>
<thead>
<tr>
<th>Region</th>
<th>Bacterium isolated</th>
<th>No. of BHS*</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hells Canyon</td>
<td>B. trehalosi</td>
<td>27</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>P. multocida</td>
<td>35</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>M. haemolytica</td>
<td>41</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>59</td>
</tr>
<tr>
<td>Other regions</td>
<td>B. trehalosi</td>
<td>54</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>P. multocida</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>82</td>
</tr>
</tbody>
</table>

* Some BHS were positive for different combinations of bacterial species.

in particular, the species M. haemolytica, B. trehalosi, and P. multocida (6–10, 12–15, 20). Bacteria from the lungs of sick and dead BHS are routinely isolated, characterized, and archived at the Caine Veterinary Teaching Center at Caldwell, ID. Analysis of these isolates over several years revealed that B. trehalosi has been isolated from pneumonic lungs of BHS at a much higher frequency than M. haemolytica (Table 1). However, >85% of B. trehalosi isolated from BHS do not produce the Lkt (28, 32). Furthermore, M. haemolytica has been shown to consistently cause severe bronchopneumonia and death of BHS under experimental conditions (10, 14, 23). These observations prompted us to investigate whether B. trehalosi outcompetes or otherwise inhibits the growth of M. haemolytica.

Growth patterns of B. trehalosi and M. haemolytica in liquid cultures. In vitro growth curves show very clearly that B. trehalosi grows faster than M. haemolytica and achieves a higher final cell density when grown in monocultures (Fig. 1) The estimated doubling time for B. trehalosi was ~10 min compared to ~27 min for M. haemolytica. The final CFU count at 24 h was 3 logs lower for M. haemolytica compared to B. trehalosi (Fig. 1). Incubation of either species beyond 24 h in the same culture media resulted in rapid decline in the number of live bacteria (not shown). The CFU of M. haemolytica/ml was significantly lower than that of B. trehalosi at all time points (P < 0.0002), except at the time of initial culture inoculation.

Differential detection of B. trehalosi from M. haemolytica in cocultures. One of the intriguing questions in BHS pneumonia concerns which of the bacterial pathogens isolated from BHS is responsible for disease progression and death. The inability to consistently identify a single pathogen from pneumonic BHS lungs complicates the identification of the bona fide etiological agent or agents of pneumonia. The results of experimental inoculation studies reveal that, unlike many other bacterial pathogens, M. haemolytica alone can cause fatal pneumonia in BHS (10, 14, 23). As described in the previous section, when grown in vitro B. trehalosi has a faster growth rate and a higher final CFU count compared to M. haemolytica, and these findings are consistent with the idea that B. trehalosi overgrows M. haemolytica in pneumonic lungs. When grown as a coculture the dynamics of these populations change dramatically. By conventional methods based on colony morphology, the ability to ferment arabinose or trehalose, and oxidase and catalase activity, M. haemolytica was detected during early hours of coculture but not beyond 6 h (data not shown). In contrast, B. trehalosi was consistently isolated at all of the time points (1 to 24 h) in very high numbers. Serially diluted bacterial samples at each time point were grown on BHI agar and at least 30 colonies were screened by multiplex PCR. Consistent with the results from morphological and biochemical characterization, we could detect M. haemolytica only during early hours of cocultures. As expected, B. trehalosi was positive at all of the time points. We have examined 50 M. haemolytica and 50 B. trehalosi isolates of BHS and domestic sheep origin to evaluate the specificity and sensitivity of the multiplex PCR. Except one isolate (which was identified as M. haemolytica by biochemical tests), there were no discrepancy between culture identification methods and PCR, indicating that the multiplex PCR we developed in the present study is specific and can differentiate the two species. When multiplex PCR assay was performed with direct culture broth (rather than with individual colonies), M. haemolytica could be detected after 24 h of coculture (Fig. 2). This observation indicated the presence of viable M. haemolytica or DNA in cocultures at 24 h but, if viable, there were too few cells available for detection by conventional microbiological methods and colony PCR assay. To circumvent this problem we cocultured Rifr B. trehalosi and

![FIG. 1. B. trehalosi exhibits a higher growth rate than M. haemolytica. The CFU for B. trehalosi (○) and M. haemolytica (●)/ml when grown in BHI broth as monocultures and the CFU of Rifr B. trehalosi (△) and Ampr M. haemolytica (▲)/ml when grown in BHI broth as cocultures were determined. The results are the mean CFU from three independent experiments (± the standard deviation). The starting culture (P > 0.05) and 1-h (P > 0.02) CFU counts were equivalent for these experiments. All other comparisons between B. trehalosi and M. haemolytica were statistically different (P < 0.0002).](image-url)

![FIG. 2. Multiplex PCR assays detect M. haemolytica in B. trehalosi/M. haemolytica cocultures, when colony PCR and bacteriological assays are negative. Lane 1, B. trehalosi monoculture (144 bp); lane 2, M. haemolytica monoculture (267 bp); lanes 3 to 7, B. trehalosi and M. haemolytica in cocultures collected after 0, 6, 12, 24, and 30 h of culture, respectively; lane 8, molecular weight markers. The results of one representative experiment out of three are shown.](image-url)
Amp\(^r\) \textit{M. haemolytica} and serially diluted samples collected at multiple time points. Samples were placed on either rifampin or ampicillin BHI agar plates. The antibiotic-resistant strains used in this experiment did not differ in their rate of growth or final CFU at 24 h when grown as monocultures. Both strains were then cultivated in six-well plates with either a 0.4- or a 0.8-μm-pore-size filter to separate cells and with Rif\(^r\) \textit{B. trehalosi} placed in the top chamber and Amp\(^r\) \textit{M. haemolytica} placed in the bottom chamber. Samples were analyzed for CFU/ml after 24 h of culture on ampicillin and rifampin plates. Compared to the expected number of Amp\(^r\) \textit{M. haemolytica} grown in monoculture, there was a significant reduction in the number of Amp\(^r\) \textit{M. haemolytica} (P < 0.0001) when cultivated with 0.8-μm-pore-size filters where there was clear migration of both species between top and bottom wells. The results are the mean CFU counts from three independent experiments.

\textbf{FIG. 3.} \textit{B. trehalosi} inhibits the growth of \textit{M. haemolytica} by a proximity-dependent mechanism. Amp\(^r\) \textit{M. haemolytica} (□) and Rif\(^r\) \textit{B. trehalosi} (■) were cultured in six-well culture plates to establish baseline CFU/ml counts at 0 and 24 h when grown as monocultures. Both strains were then cultivated in six-well plates with either a 0.4- or a 0.8-μm-pore-size filter to separate cells and with Rif\(^r\) \textit{B. trehalosi} placed in the top chamber and Amp\(^r\) \textit{M. haemolytica} placed in the bottom chamber. Samples were analyzed for CFU/ml after 24 h of culture on ampicillin and rifampin plates. Compared to the expected number of Amp\(^r\) \textit{M. haemolytica} grown in monoculture, there was a significant reduction in the number of Amp\(^r\) \textit{M. haemolytica} (P < 0.0001) when cultivated with 0.8-μm-pore-size filters where there was clear migration of both species between top and bottom wells. The results are the mean CFU counts from three independent experiments.

\textbf{Evaluation of culture supernatants for bactericidal activity.} Because \textit{B. trehalosi} inhibited the growth of \textit{M. haemolytica} in cocultures, we next determined whether this inhibition was mediated by lytic bacteriophages, bactericidal compounds, or direct cell-to-cell contact. A diverse array of bacteriophages has been isolated from \textit{B. trehalosi}, \textit{M. haemolytica}, and \textit{P. multocida} strains (1, 11), and some of the bacteriophage isolates were able to form plaques with indicator strains of the same species (11). We did not observe any plaque formation with either individual \textit{B. trehalosi}, \textit{M. haemolytica}, or coculture supernatants on \textit{B. trehalosi} or \textit{M. haemolytica} agar plates at 30, 37, and 42°C (Table 2). This observation suggested the absence of lytic bacteriophages in the culture supernatant fluids under our experimental conditions. No attempts were made to identify bacteriophages (if there were any in culture supernatant fluids) by electron microscopy due to the lack of lytic phase activity. Therefore, we could not rule out the presence of noninfectious prophages in the culture supernatant fluids.

\begin{table}[h]
\centering
\caption{Bactericidal activity of \textit{M. haemolytica} and \textit{B. trehalosi} culture supernatant fluids\textsuperscript{a}}
\begin{tabular}{lll}
\hline
Culture supernatant & Bactericidal activity & Plaque formation \\
\hline
\textit{M. haemolytica} & – & – \\
\textit{B. trehalosi} & – & – \\
Coculture & – & – \\
\hline
\end{tabular}
\textsuperscript{a}Five different \textit{M. haemolytica} and \textit{B. trehalosi} strains or isolates were examined by using a lytic phase assay at 30, 37, and 42°C. –, lack of bactericidal activity or plaque formation.
\end{table}
in any other bacterial species, although a potential BamA homologue has been annotated for the *Pasteurella* species (18). It is also possible that quorum-sensing systems may be triggered during coculture that causes *M. haemolytica* to alter growth phase (22, 27). To determine whether related mechanisms are relevant to our system, contact-dependent inhibition assays were performed using cell culture inserts with PET membranes as described in Materials and Methods. When *B. trehalosi* in the upper chamber was separated from *M. haemolytica* in the lower chamber by filters with a pore size of 0.4 μm, the number of *B. trehalosi* in the upper chamber was not significantly different from that in cultures containing *B. trehalosi* alone (Fig. 3). The number of *M. haemolytica* in the lower chamber was lower than that in cultures containing *M. haemolytica* alone, but this difference may be attributable to the presence of some *B. trehalosi* in the lower chamber (Fig. 3). The presence of *B. trehalosi* in the lower chamber was unexpected because most bacteria cannot pass through 0.4-μm pores. In order to ensure that there was no cross-contamination, the following experiments were performed. When *M. haemolytica* was placed in the upper chamber and *B. trehalosi* was placed in the lower chamber separated by 0.4-μm-pore-size membranes, a small number of *M. haemolytica* (~10^2 CFU/ml) were detected in the lower chamber, while *B. trehalosi* was not detected in the upper chamber (data not shown).

Similar results were observed in repeated experiments confirming the lack of cross-contamination. Thus, it appears that the chambers allow some passage of cells, presumably due to manufacturing defects. Nevertheless, this experiment was consistent with our earlier conclusion that *M. haemolytica* numbers decreased when cultured in close proximity to *B. trehalosi*. This result is consistent with either a contact-dependent or proximity-dependent mechanism where the latter would involve bacteriocins or quorum-sensing systems that are only functional at very high concentrations achieved by labile proteins that are in high concentration proximal to the intersecting cells. When larger pore sizes (8.0 μm) were used, the concentrations of *B. trehalosi* were identical in both the upper and the lower chambers and were not different from that in cultures containing *B. trehalosi* alone (Fig. 3). This result indicated that *B. trehalosi* was able to easily cross the membrane boundary. The number of *M. haemolytica* in both chambers was significantly lower than the number in cultures containing *M. haemolytica* alone, a finding consistent with growth inhibition mediated by a contact-dependent or proximity-dependent mechanism.

In summary, we have clearly demonstrated here that *B. trehalosi* induces inhibition of *M. haemolytica* and that the inhibition is mediated by a mechanism that requires close proximity between the inhibitor (*B. trehalosi*) and the target (*M. haemolytica*). This mechanism could be explained by the presence of soluble signaling molecules (e.g., quorum sensing) or bacteriocins, although we found no evidence for such molecules with our assays, and these proteins would probably be quite labile to be undetected in the culture supernatant. Alternatively, such compounds might only be effective given very high concentrations achieved in close proximity but, presumably, such proximity-dependent concentrations would also be difficult to achieve while cultures are being shaken at 200 rpm. Thus, we submit that the more parsimonious explanation involves a contact-dependent mechanism. Regardless of the mechanisms involved, *B. trehalosi* can overgrow, while simultaneously inhibiting *M. haemolytica* growth, and if these patterns reflect in vivo conditions, then these results are consistent with the failure to routinely isolate *M. haemolytica* from pulmonary lungs from sick or dead BHS. Contact-dependent inhibition was first described among *E. coli* strains, but if a similar mechanism explains our observations it will be the first report of contact-dependent inhibition between two different bacterial species. The molecular basis underlying the inhibition of growth of *M. haemolytica* is currently under investigation in our laboratory.

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