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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 pneumonic 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 a 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 pneumonic 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 (89010807N [21]) and other BHS isolates, as well as *B. trehalosi* serotype T10 (ATCC 33374) and other BHS isolates, were individually cultured 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 (Amp^r) *M. haemolytica* and rifampin-resistant (Rif^r) *B. trehalosi* were used in subsequent studies.

Isolation of bacteria from pneumonic 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% CO_2 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; pleiomorphic; MacConkey, urea, and indole negative; oxidase, nitrate, glucose, and sucrose positive; and xylose or trehalose positive. Each isolate was biovariant-typed 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 (~10^6 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. After overnight growth, bacterial colonies were counted and plotted against time.

Plaque 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 Life Sciences, East Hills, NY). A total of 100 μl of *M. haemolytica* (10^6 CFU) and 300 μl of culture supernatant fluids were mixed and incubated at 37°C for 20 min to facilitate adsorption of bacteriophage (if any), after which 3 ml of BHI soft molten agar (0.7%, 45°C) was added into each tube. The tube was mixed gently and immediately poured onto preheated BHI agar plates with a swirling movement. The plates were kept at room temperature for 5 min for the soft agar to solidify and then incubated at 30, 37, and 42°C. Culture plates were examined for plaque (zone of lysis) formation for up to 24 h. *M. haemolytica* without culture supernatant fluids, but with soft molten agar, was used as negative control.

Bactericidal assay. The presence of any bactericidal compounds in *B. trehalosi* and coculture supernatant fluids was evaluated by solid BHI agar and broth assays, described below. To minimize loss of bactericidal compounds (if any) during filtration, culture supernatants were filtered through low protein binding filters (Acrodisc 0.2- or 0.45-μm-pore-size Supor membrane syringe filters; Pall Life Sciences). BHI broth (100 μl) containing 10^6 CFU of *M. haemolytica* was gently mixed with 3 ml of BHI soft molten agar and poured onto preheated BHI agar plates with a swirling movement. Plates were kept at room temperature for 5 min. Discs soaked with filtered culture supernatant fluids were placed onto the solidified soft agar and incubated at 37°C. Plates were examined up to 24 h for signs of inhibition, as revealed by clear zones around the discs. Putative bacteriocin(s) can be precipitated from culture supernatant by using ammonium sulfate (4). Putative bacteriocins in *M. haemolytica* and *B. trehalosi* culture supernatants (400 ml) were precipitated with 40% ammonium sulfate and dialyzed against phosphate-buffered saline (pH 7.2). Portions (100 to 150 μg) of solubilized proteins were tested for bacteriocin activity on *M. haemolytica* plates by the spot-on-lawn assay. A bactericidal assay was also performed using broth culture. Filter-sterilized culture supernatant fluid (5 ml) was mixed with equal volumes of 2× BHI broth containing *M. haemolytica* (10^6 CFU) and cultured for 24 h at 37°C (200 rpm). *M. haemolytica* CFU counts were determined as described above. *M. haemolytica* without any culture supernatant fluids, but with BHI broth, served as the negative control. To determine whether the filter membrane has any undesirable effect on the putative bactericidal compounds, a direct inhibitory assay was performed as follows. *M. haemolytica* in soft agar was overlaid onto BHI agar as described earlier. Then, BHI containing 10^5 CFU (10 μl) of actively growing *B. trehalosi* was placed onto the soft agar, followed by incubation at 37°C for up to 24 h. The plates were examined for zones of inhibition, indicated by a clear halo around the colony.

Detection of *M. haemolytica* and *B. trehalosi* in cocultures by conventional culture techniques. Overnight cultures of *B. trehalosi* and *M. haemolytica* were diluted in BHI broth and added into 10 ml of BHI broth (~10:1 inhibitor/target ratio; that is, *B. trehalosi* [~10^7 CFU/ml] to *M. haemolytica* [~10^6 CFU/ml]). The same numbers of *B. trehalosi* and *M. haemolytica* were individually inoculated into 10 ml of BHI broth as well. All three cultures were incubated at 37°C for 24 h (200 rpm), and samples were collected at different time points (1, 3, 6, 12, and 24 h). One set of samples was submitted to the Washington Animal Disease Diagnostic Laboratory at Washington State University to analyze independently where standard bacteriological methods are used to identify *B. trehalosi* and *M. haemolytica*. The other set of samples were serially diluted and plated on BHI agar plates, followed by colony PCR (described below). In the same coculture experiments, antibiotic sensitive bacteria were replaced with Amp^r *M. haemolytica* and Rif^r *B. trehalosi* strains. Serially diluted bacteria from the individual (mono-) and cocultures collected at different time points were placed 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 (*gcp*; GenBank accession numbers AY839677 and AY839681) and manganese-dependent superoxide dismutase (*sodA*; GenBank accession numbers AY702551 and AY702549) 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 *gcp* forward primer MhgcpF (5′-AGA GGC CAA TCT GCA AAC CTC G-3′) and reverse primer MhgcpR (5′-GTT CGT ATT GCC CAA CGC CG-3′), and *B. trehalosi*-specific *sodA* forward primer BtsodAF (5′-GCC TGC GGA CAA ACG TGT TG-3′) and reverse primer BtsodAR (5′-TTT CAA CAG AAC CAA AAT CAC GAA TG-3′). The multiplex PCR was carried out in a final volume of 50 μl with GoTaq PCR SuperMix (Promega, Inc., 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 (*gcp*, 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 polyethylene terephthalate (PET) track-etched membrane cell culture inserts (BD Falcon; BD Biosciences, Franklin Lakes, NJ) to further characterize the observed growth inhibition. Placing these inserts in six-well cell culture plates creates upper and lower chambers. Approximately 10^8 CFU of Rif^r *B. trehalosi* in 10 μl of BHI were added into the upper chambers containing 2.5 ml of antibiotic-free BHI, and ~10^7 CFU of Amp^r *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^r *B. trehalosi* and Amp^r *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 pneumonic BHS lungs. Bacteria associated with BHS pneumonia are members of the genera *Mannheimia* and *Pasteurella*,

TABLE 1. Bacteria isolated from pneumonic lungs of BHS

Region	Bacterium isolated	No. of BHS[a]	% Total
Hells Canyon	*M. haemolytica*	10	17
	B. trehalosi	27	46
	P. multocida	35	59
Total		59	
Other regions	*M. haemolytica*	41	50
	B. trehalosi	54	66
	P. multocida	25	30
Total		82	

[a] Some BHS were positive for different combinations of bacterial species.

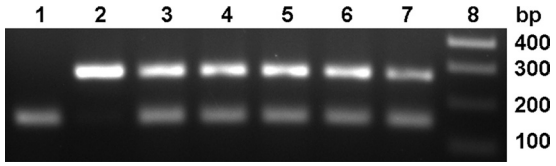

FIG. 2. Multiplex PCR assays detect *M. haemolytica* in *B. trehalosi*/*M. haemolytica* cocultures, when colony PCR and bacteriological assays are negative. Lane 1, *B. trehalosi* monoculture (144 bp); lane 2, *M. haemolytica* monoculture (267 bp); lanes 3 to 7, *B. trehalosi* and *M. haemolytica* in 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.

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.

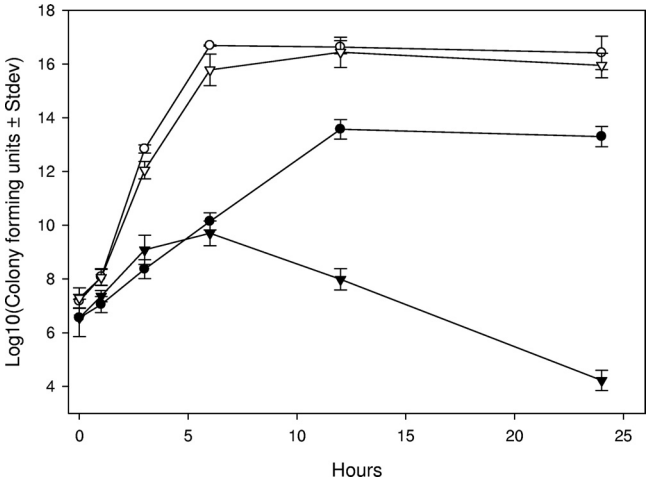

FIG. 1. *B. trehalosi* exhibits a higher growth rate than *M. haemolytica*. The CFU for *B. trehalosi* (○) and *M. haemolytica* (●)/ml when grown in BHI broth as monocultures and the CFU of Rif^r *B.* trehalosi (△) and Amp^r *M. haemolytica* (▲)/ml when grown in BHI broth as cocultures were determined. 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 ($P < 0.0002$).

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 Rif^r *B. trehalosi* and

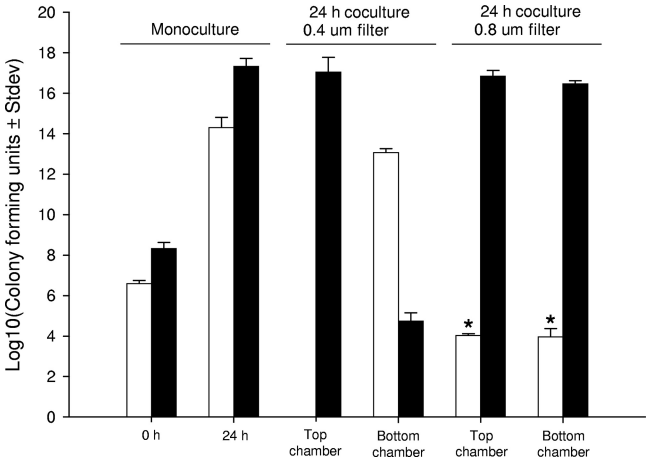

FIG. 3. *B. trehalosi* inhibits the growth of *M. haemolytica* by a proximity-dependent mechanism. Amp^r *M. haemolytica* (□) and Rif^r *B. trehalosi* (■) were cultured in six-well culture plates to establish baseline CFU/ml counts at 0 and 24 h when grown as monocultures. Both strains were then cultivated in six-well plates with either a 0.4- or a 0.8-μm-pore-size filter to separate cells and with Rif^r *B. trehalosi* placed in the top chamber and Amp^r *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 *M. haemolytica* grown in monoculture, there was a significant reduction in the number of Amp^r *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.

Amp^r *M. haemolytica* and serially diluted samples collected at multiple time points. Samples were placed on both rifampin and 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 (Fig. 3). When cocultured, *M. haemolytica* growth was not affected for up to 4 h, after which growth slowed and began to decline rapidly from 6 to 24 h (Fig. 1). Between 12 and 24 h there was an ~9-log decline in the number of viable *M. haemolytica* compared to what would be expected in monoculture. The decline was observed initially as *B. trehalosi* was transitioning from log to stationary phase, which is consistent with *B. trehalosi* inhibiting the growth of *M. haemolytica* when cocultured. Collectively, these findings suggest that it is possible that the failure to consistently detect *M. haemolytica* in BHS pneumonic lungs is due to both overgrowth of *B. trehalosi* and simultaneous reduction in cell density of *M. haemolytica*. The failure of conventional culture methods to detect *M. haemolytica* in these samples could be due to the absence of this organism or its presence in insufficient numbers to be detected by the conventional methods. The problem of differential detection is compounded by inevitable delays between sample collection under field conditions and laboratory analysis. This delay may contribute to an opportunity for *B. trehalosi* to further outgrow and inhibit growth and viability of *M. haemolytica*. From a clinical diagnostic perspective, conventional culture methods are probably not sensitive enough to consistently detect *M. haemolytica* in field samples.

Evaluation of culture supernatants for bactericidal activity. Because *B. trehalosi* inhibited the growth of *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 *B. trehalosi*, *M. haemolytica*, and *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 *B. trehalosi*, *M. haemolytica*, or coculture supernatants on *B. trehalosi* or *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 phage activity. Therefore, we could not rule out the presence of noninfectious prophages in the culture supernatant fluids. To identify any bactericidal compounds in the culture supernatant fluids, filter-sterilized *B. trehalosi* supernatant fluid-soaked discs or drops (100 to 150 μg of precipitated proteins in 10 μl) were placed in contact with *M. haemolytica*, but neither of them produced detectable growth inhibition (Table 2). To determine whether the association of both bacteria is needed for *B. trehalosi* to secrete inhibitory compounds, the same assays were performed with coculture supernatant fluids, but there was no inhibition of *M. haemolytica* growth on agar plates. Doubling the concentration of culture supernatant did not change this outcome, indicating that *B. trehalosi* does not produce any growth inhibitory compounds (data not shown). Direct plating of *B. trehalosi* on *M. haemolytica* plates was also performed to rule out the presence of inhibitory compounds with a very short half-life or low stability under culture conditions, but no inhibition was detected (data not shown). Taken together, these observations clearly indicate that the inhibition of *M. haemolytica* growth in cocultures was not due to lytic bacteriophage or secreted bactericidal compounds originating from *B. trehalosi*.

***B. trehalosi* inhibits *M. haemolytica* growth by a proximity-dependent mechanism.** The failure of the culture supernatant fluid from *B. trehalosi* to inhibit *M. haemolytica* growth prompted us to investigate other mechanism(s) of growth inhibition. Aoki et al. (2) have described a novel phenomenon called "contact-dependent growth inhibition" whereupon some strains of *Escherichia coli* inhibit the growth of other strains *E. coli* (target). The inhibitory and target *E. coli* strains were genetically different from each other (2), and inhibition involved a specific ligand interaction between CdiAB from the inhibitory strains and BamA (YaeT), which is displayed on the surface membrane of target strains (3). To date, this phenomenon of contact-dependent inhibition has not been identified

TABLE 2. Bactericidal activity of *M. haemolytica* and *B. trehalosi* culture supernatant fluids[a]

Culture supernatant	Bactericidal activity	Plaque formation
M. haemolytica	–	–
B. trehalosi	–	–
Coculture	–	–

[a] Five different *M. haemolytica* and *B. trehalosi* strains or isolates were examined by using a lytic phage assay at 30, 37, and 42°C. –, lack of bactericidal activity or plaque formation.

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* ($\sim 10^2$ 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 interacting 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 pneumonic 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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