Proximity-Dependent Inhibition of Growth of *Mannheimia haemolytica* by *Pasteurella multocida*

Jegarubee Bavananthasivam,^a Rohana P. Dassanayake,^a Abirami Kugadas,^a Sudarvili Shanthalingam,^a Douglas R. Call,^a Donald P. Knowles,^{a,b} and Subramaniam Srikumaran^a

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, USA,^a and Animal Disease Research Unit, USDA Agricultural Research Service, Washington State University, Pullman, Washington, USA^b

Mannheimia haemolytica, *Pasteurella multocida*, and *Bibersteinia trehalosi* have been identified in the lungs of pneumonic bighorn sheep (BHS; *Ovis canadensis*). Of these pathogens, *M. haemolytica* has been shown to consistently cause fatal pneumonia in BHS under experimental conditions. However, *M. haemolytica* has been isolated by culture less frequently than the other bacteria. We hypothesized that the growth of *M. haemolytica* is inhibited by other bacteria in the lungs of BHS. The objective of this study was to determine whether *P. multocida* inhibits the growth of *M. haemolytica*. Although in monoculture both bacteria exhibited similar growth characteristics, in coculture with *P. multocida* there was a clear inhibition of growth of *M. haemolytica*. The inhibition was detected at mid-log phase and continued through the stationary phase. When cultured in the same medium, the growth of *M. haemolytica* was inhibited when both bacteria were separated by a membrane that allowed contact (pore size, 8.0 μm) but not when they were separated by a membrane that limited contact (pore size, 0.4 μm). Lytic bacteriophages or bactericidal compounds could not be detected in the culture supernatant fluid from monocultures of *P. multocida* or from *P. multocida*-*M. haemolytica* cocultures. These results indicate that *P. multocida* inhibits the growth of *M. haemolytica* by a contact- or proximity-dependent mechanism. If the inhibition of growth of *M. haemolytica* by *P. multocida* occurs *in vivo* as well, it could explain the inconsistent isolation of *M. haemolytica* from the lungs of pneumonic BHS.

Pneumonia is an important disease of domestic and wild ruminants (17, 19, 27, 38). The morbidity and mortality, however, varies among the different ruminant species. Bighorn sheep (BHS; *Ovis canadensis*) are particularly susceptible to pneumonia, which is considered the primary disease responsible for the drastic decline of these animals in North America, from an estimated two million animals at the beginning of the 19th century to less than 70,000 at this time (8, 37). The bacteria detected in the pneumonic lungs of BHS include *Mannheimia haemolytica*, *Bibersteinia trehalosi*, *Pasteurella multocida*, and *Mycoplasma ovipneumoniae*. *M. haemolytica* produces an exotoxin known as leukotoxin (Lkt). Although it is cytolytic to all subsets of ruminant leukocytes, polymorphonuclear leukocytes (PMNs) are the most susceptible subset (10, 24). Lkt-induced cytolysis of PMNs is responsible for the acute inflammation and lung injury characteristics of this disease. Based on the fact that *lktA* deletion mutants of *M. haemolytica* A1 cause no mortality in BHS (12) and reduced mortality and mild lung lesions in calves, Lkt has been accepted as the most important virulence factor of *M. haemolytica* (22, 30, 34). More than 90% of *B. trehalosi* isolates of BHS origin do not produce leukotoxin (33, 39). *P. multocida* has not been shown to produce virulence factors that can cause the type of lesions observed in pneumonic BHS lungs, particularly dead and dying PMNs and macrophages. Hence, *B. trehalosi* and *P. multocida* are unlikely to be the major pathogens of pneumonia in BHS (35). Experimental inoculation studies have shown that *M. ovipneumoniae* does not cause fatal pneumonia in BHS lambs (5). Nevertheless, it can predispose adult BHS to *M. haemolytica* pneumonia (6, 14). It is not clear whether *M. ovipneumoniae* is a necessary predisposing agent for BHS pneumonia. *M. haemolytica* has been shown to consistently cause fatal pneumonia in BHS under experimental conditions (12, 17, 19, 29), but *M. haemolytica* has been isolated from pneumonic lungs much less frequently than the other organisms (13). How-

ever, species-specific PCR assays have identified *M. haemolytica* in the lung tissues of pneumonic BHS much more frequently than the other organisms (31, 33). Consequently, we hypothesized that the growth of *M. haemolytica* is inhibited by other bacteria in the lungs. *B. trehalosi* has been shown to inhibit the growth of *M. haemolytica* *in vitro* (13). Therefore, the objectives of the present study were (i) to characterize the *in vitro* growth curves of *M. haemolytica* and *P. multocida*, (ii) to determine whether *P. multocida* inhibits the growth of *M. haemolytica* and, if it does, (iii) to characterize the possible mechanism(s) of inhibition.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Four field isolates of *P. multocida* (BHS 3272, 07/1920/139, 96/65/2, and 95/1545/4) and four isolates of *Moraxella bovoculi* obtained from the Washington Animal Disease Diagnostic Laboratory were used in the present study. An ampicillin-resistant leukotoxin (*lktA*) deletion mutant of *M. haemolytica* A1 previously developed by Murphy et al. (28) and an *lktA*⁺ *M. haemolytica* isolate (10/2/3) previously tagged with green fluorescent protein by us (25) were also used in the present study. From the freezer stock, all isolates of *P. multocida*, *M. haemolytica*, and *M. bovoculi* were plated in brain heart infusion (BHI) agar plates and incubated overnight at 37°C. Single colonies from BHI plates were cultured in 5 ml of BHI broth in individual tubes overnight at 37°C with shaking (225 rpm). These bacterial cultures were then spread on individual BHI agar plates containing ampicillin (25 $\mu\text{g/ml}$), rifampin

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Address correspondence to Subramaniam Srikumaran, ssrikumaran@vetmed.wsu.edu.

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(10 µg/ml), nalidixic acid (30 µg/ml), or tetracycline (15 µg/ml). Each antibiotic-resistant isolate was spread on other antibiotic-containing BHI agar plates to determine resistance to other antibiotics. In this manner, four rifampin-resistant (Rif^r) *P. multocida* isolates (BHS 3272, 07/1920/139, 96/65/2, and 95/1545/4), one nalidixic acid-resistant (Nal^r) *P. multocida* isolate (07/1920/213), and one Nal^r *M. haemolytica* isolate (91N-10) were obtained and stored as frozen stocks in 30% glycerol in BHI at -80°C. These antibiotic-resistant isolates were used here because of the ease of detection of these strains (by growth in appropriate antibiotic-containing media).

In order to determine the specificity of inhibition of growth of *M. haemolytica* by *P. multocida*, the coculture experiment was also performed with killed *P. multocida*. For this experiment, approximately 5×10^7 CFU of *P. multocida* were killed by incubating with 1 ml of 70% isopropyl alcohol for 1 h. The bacteria were then washed and resuspended in 1 ml of BHI.

CFU assay. The number of CFU of *P. multocida*, *M. haemolytica*, and *M. bovoculi* in monocultures and cocultures were determined by CFU assay as described previously by us (13). The *M. bovoculi* strain used here was not resistant to any antibiotic. The number of CFU of *M. bovoculi* in coculture was determined by subtracting the number of CFU of *M. haemolytica*, which was determined by enumerating the number of CFU of this bacterium on ampicillin-resistant BHI plates.

Proximity-dependent inhibition assay. This assay was performed as previously described (13). To determine whether *P. multocida*-mediated growth inhibition of *M. haemolytica* was consistent with contact- or proximity-dependent inhibition, cell culture inserts with two different pore sizes (0.4 and 8.0 µm) were used. Bactericidal compounds and phages are able to diffuse through 0.4-µm pores. Generally, bacteria do not pass through 0.4-µm pores, but they can readily pass through 8.0-µm pores. The 0.4- and 8.0-µm-pore-size polyethylene terephthalate (PET) track-etched membrane cell culture inserts (BD Falcon; BD Biosciences, Franklin Lakes, NJ) were placed in six-well cell culture plates creating upper and lower chambers. Approximately 10^6 CFU of Rif^r *P. multocida* in 10 µl of BHI were added into the upper chambers containing 2.5 ml of antibiotic-free BHI, and $\sim 10^5$ CFU of ampicillin-resistant (Amp^r) *M. haemolytica* (10:1 inhibitor/target ratio) in 10 µl of BHI were added into the lower chambers containing the same volume of antibiotic-free BHI. Evaporation of the culture medium was minimized by covering the culture plates with the lids and wrapping with parafilm. The plates were incubated at 37°C with shaking at 100 rpm. Aliquots were collected from both upper and lower chambers at 24 h, serially diluted with BHI broth, and placed on each type of agar plates (ampicillin- or rifampin-containing BHI agar plates). Rif^r *P. multocida* and Amp^r *M. haemolytica* individually cultured in six-well culture plates with the same culture volume were used as treatment controls.

Bactericidal assay. Bactericidal compounds in *P. multocida* monoculture and *P. multocida*-*M. haemolytica* coculture supernatant fluids were collected at 6 and 24 h postinoculation and evaluated by solid BHI agar and broth assays, as described previously (13). Culture supernatant fluids were filtered through low protein binding filters (Acrodisc 0.2- or 0.45-µm-pore-size Supor membrane syringe filters; Pall Life Sciences) to minimize the loss of bactericidal compounds (if any) during filtration. *M. haemolytica* ($\sim 10^6$ CFU in 100 µl of BHI broth) gently mixed with BHI soft molten agar (3 ml) was poured onto preheated (37°C) BHI agar plates with a swirling movement and kept at room temperature for 5 min. A total of 20 µl of culture supernatant fluid from *P. multocida* or *M. haemolytica* monoculture or *P. multocida* and *M. haemolytica* coculture was placed on solidified soft agar, followed by incubation overnight at 37°C. The plates were examined the following day for zones of inhibition on the agar plates. This assay was repeated with 10 µl of *P. multocida* culture instead of the culture supernatant fluid. In another experiment $\sim 10^6$ CFU of *M. haemolytica* in 5 ml of 2× BHI broth was mixed with 5 ml of mono- or coculture filter-sterilized supernatant fluid, followed by incubation for 24 h at 37°C with shaking (225 rpm). Samples were then collected, and CFU

FIG 1 The antibiotic-resistant isolates of *M. haemolytica* and *P. multocida* exhibit comparable growth rates as their respective parent strains. The CFU/ml for antibiotic-sensitive (◇) and antibiotic-resistant (△) *M. haemolytica* and antibiotic-sensitive (○) and antibiotic-resistant (□) *P. multocida* when grown in BHI broth as monocultures were determined. The results are the mean CFU/ml from three independent experiments ($\pm$ the standard deviations [SD]). There was no statistically significant difference ($P > 0.05$ based on repeated measures of ANOVA) between the CFU/ml for the antibiotic-resistant and antibiotic-sensitive isolates.

assay was performed to calculate the number of bacteria in each of the culture tubes. *M. haemolytica* without the culture supernatant fluid was used as a negative control.

If a soluble inhibitor is involved, it is possible that its concentration in the supernatant fluid is too low to observe the inhibition. Therefore, the supernatant fluids were concentrated by ammonium sulfate precipitation (4). Approximately 100 to 150 µg of 40% ammonium sulfate-precipitated proteins was used for the detection of bacteriocin activity on *M. haemolytica* plates by a "spot-on-lawn assay." *M. haemolytica* with BHI broth alone was used as a negative control.

PCR. Multiplex PCR assay was performed to confirm the identity of *M. haemolytica* and *P. multocida* colonies in each plate and broth culture. The previously designed *M. haemolytica* species-specific forward primer MhgcF (5'-AGA GGC CAA TCT GCA AAC CTC G-3') and reverse primer MhgcR (5'-GTT CGT ATT GCC CAA CGC CG-3') (13) and the *P. multocida* species-specific primer KMT1T7 (5'-ATC CGC TAT TTA CCC AGT GG-3') and primer KMT1SP6 (5'-GCT GTA AAC GAA CTC GCC AC-3') (36) were used. PCR was performed with GoTaq green master mix (Promega, Inc., Madison, WI) in a 25-µl final volume as described previously (13).

Statistical analysis. Bacterial numbers were calculated and are expressed as the mean $\log_{10}$ CFU/ml of culture media $\pm$ the standard deviation determined at different time points. The data were statistically analyzed by repeated-measures analysis of variance (ANOVA) and Student *t* test using GraphPad online software (GraphPad, La Jolla, CA). A *P* value of <0.05 was considered statistically significant.

RESULTS

***P. multocida* inhibits the growth of *M. haemolytica* in coculture.** When *P. multocida* and *M. haemolytica* were cultured individually, both species exhibited similar growth characteristics and yielded overlapping growth curves (data not shown). The average doubling time for *M. haemolytica* and *P. multocida* was 26 min. Rif^r *P. multocida* and Amp^r *M. haemolytica* strains did not differ from the respective wild-type strains with respect to growth in culture (Fig. 1).

When Rif^r *P. multocida* and Amp^r *M. haemolytica* A1 *lktA* deletion mutants were cocultured, the CFU count of Amp^r *M. haemolytica* began to decrease at 3 h and continued to decrease through the stationary phase (Fig. 2). At 24 h, the CFU count of Amp^r *M. haemolytica* in coculture was 4 logs lower than that in

FIG 2 *P. multocida* inhibits the growth of *M. haemolytica* A1 in coculture *in vitro*. The CFU/ml for *P. multocida* (BHS3272) when grown as monoculture (◇) or coculture (△) with *M. haemolytica* were determined at the indicated time points. Similarly, the CFU/ml for *M. haemolytica* when grown as monoculture (○) or coculture (□) with *P. multocida* were determined at the indicated time points. The results are the mean CFU from three independent experiments (± the SD). The CFU/ml for *M. haemolytica* in monoculture and *M. haemolytica* in coculture are statistically significantly different at 6, 12, and 24 h postinfection (*, $P < 0.0001$ based on repeated measures of ANOVA). There was no statistically significant difference between the CFU/ml for *P. multocida* in monoculture and *P. multocida* in coculture at all time points ($P > 0.05$).

monoculture. In contrast, the CFU count of Rif^r *P. multocida* in coculture was almost identical to that in monoculture. A similar inhibition of growth was observed with another Amp^r *M. haemolytica* strain of unknown serotype (Fig. 3), except that the difference in CFU counts between monoculture and coculture was 3 logs at 24 h.

To examine the generality of the inhibitory effect of *P. multocida* on *M. haemolytica*, we performed coculture experiments involving different field isolates of Rif^r Nal^r *P. multocida* and Amp^r Nal^r *M. haemolytica*. Similar results were obtained with all of these pairs of *P. multocida* and *M. haemolytica* (Table 1). The reduction in the CFU counts of the *M. haemolytica* isolates in coculture ranged from 2 to 4 logs, and the reduced CFU counts were significantly different from the CFU counts of *M. haemolytica* in monoculture ($P < 0.0001$). The CFU counts of *P. multocida* strains in

FIG 3 *P. multocida* inhibits the growth of *M. haemolytica* 10/2/3 in coculture *in vitro*. The CFU/ml for *P. multocida* (96/65/2) when grown as monoculture (◇) or coculture (△) with *M. haemolytica* were determined at indicated time points. Similarly, the CFU/ml for *M. haemolytica* when grown as monoculture (○) or coculture (□) with *P. multocida* were determined at the indicated time points. The results are the mean CFU from three independent experiments (± the SD). The CFU/ml for *M. haemolytica* in monoculture and *M. haemolytica* in coculture are statistically significantly different at 6, 12, and 24 h postinfection (*, $P < 0.05$ based on repeated measures of ANOVA). There was no statistically significant difference between the CFU/ml for *P. multocida* in monoculture and *P. multocida* in coculture at all time points ($P > 0.05$).

TABLE 1 Inhibition of the growth of *M. haemolytica* by *P. multocida*

Bacterial strain combination (<i>M. haemolytica</i> strain, <i>P. multocida</i> strain) ^a	Mean no. of bacteria (log ₁₀ CFU/ml) ^b			
	<i>M. haemolytica</i>		<i>P. multocida</i>	
	Monoculture	Coculture	Monoculture	Coculture
A1 mutant, BHS3272	6.95	2.87	8.87	8.83
A1 mutant, 07/1920/139	7.21	2.68	9.29	8.65
A1 mutant, 95/1545/4	7.32	4.15	8.47	7.94
10/2/3 (gfp), 96/65/2	8.04	4.96	8.09	8.17
91N-10, 07/1920/139	8.34	5.57	9.50	8.44
91N-10, BHS3272	8.05	4.23	7.70	7.60

^a Three different isolates of *M. haemolytica* were cocultured with four different isolates of *P. multocida* in different combinations.

^b The numbers represent the log₁₀ CFU/ml of the coculture at after 24 h. The difference in the CFU/ml for *M. haemolytica* in monocultures and cocultures is statistically significant ($P < 0.0001$). Results are based on two independent experiments, except for the first two combinations of bacterial strains, which are based on three independent experiments.

coculture were not different from the CFU counts in monoculture, indicating that the growth of *P. multocida* was not inhibited by *M. haemolytica*. *P. multocida*- and *M. haemolytica*-specific PCR assays confirmed the specificity of the selective plating protocol.

To determine the specificity of the inhibition of growth of *M. haemolytica* by *P. multocida*, *M. haemolytica* was also cocultured with *M. bovoculi*. As shown in Fig. 4, the growth of *M. haemolytica* was not inhibited by *M. bovoculi*, indicating that the inhibition of growth of *M. haemolytica* by *P. multocida* was specific. Furthermore, coculture of *M. haemolytica* with killed *P. multocida* did not result in the inhibition of growth of *M. haemolytica*, suggesting the involvement of a secreted inhibitory molecule in the growth inhibition (data not shown).

***P. multocida* inhibition of *M. haemolytica* is consistent with a proximity-dependent mechanism.** We performed a proximity-dependent inhibition assay using cell culture inserts with pore-size of either 0.4 or 8.0 μm. When *P. multocida* in the upper chamber was separated from *M. haemolytica* in the lower chamber using a 0.4-μm-pore-size membrane, the CFU counts of *P. multocida* in the upper chamber and *M. haemolytica* in the lower chamber were similar to that observed in monocultures of *P. multocida* and *M.*

FIG 4 The growth of *M. haemolytica* A1 is not inhibited by *M. bovoculi* in coculture. The CFU/ml for *M. bovoculi* when grown as monoculture (—○—) or coculture (—) with *M. haemolytica* were determined at the indicated time points. Similarly, the CFU/ml for *M. haemolytica* when grown as a monoculture (dotted line) or coculture (dashed line) with *M. bovoculi* were determined at the indicated time points. The results are the mean CFU from three independent experiments (± the SD). *M. haemolytica* exhibited similar growth curves in monocultures and cocultures with *M. bovoculi*.

FIG 5 *P. multocida* inhibits the growth of *M. haemolytica* by a proximity-dependent mechanism. Amp^r *M. haemolytica* (□) and Rif^r *P. multocida* (■) were cultured in six-well culture plates to establish baseline CFU (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 8.0-µm-pore-size filter to separate cells and with Rif^r *P. multocida* placed in the top chamber and Amp^r *M. haemolytica* placed in the bottom chamber. Samples from each chamber were analyzed for CFU/ml at 24 h of culture on ampicillin- as well as rifampin-containing BHI agar 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* (*, $P < 0.0001$ [Student *t* test]) when cultivated with 8.0-µm-pore-size filters, where there was clear migration of both species between the top and bottom chambers. The results are the mean CFU counts from four independent experiments. The bars indicate the SD of the means and are based on four independent experiments.

haemolytica, respectively (Fig. 5). When these cultures were separated by using an 8.0-µm-pore-size membrane, the CFU counts of *P. multocida* in the upper and lower chambers were similar to those observed in monocultures of this bacterium ($P > 0.05$). In contrast, the average CFU counts of *M. haemolytica* in the upper and lower chambers were significantly lower than those observed in monocultures for this bacterium ($P < 0.001$). Identical results were obtained when *P. multocida* was placed in the lower chamber and *M. haemolytica* was placed in the upper chamber (data not shown). Taken together, these results indicate that *P. multocida* inhibits the growth of *M. haemolytica* by a contact- or proximity-dependent mechanism.

Inhibition of *M. haemolytica* by *P. multocida* is not mediated by observable soluble bactericidal compounds. To assess the potential for any bactericidal compounds produced by *P. multocida*, culture supernatant fluid from this bacterium was placed in contact with *M. haemolytica*, and no inhibition of growth of *M. haemolytica* was observed. It is plausible that the lack of inhibition of the growth of *M. haemolytica* could be due to the low concentration of the bactericidal compound, although even 150 µg of proteins (in 50 µl) precipitated from the culture fluid by ammonium sulfate did not inhibit the growth of *M. haemolytica*. To determine whether the coculture of both bacteria is necessary for *P. multocida* to produce the bactericidal compound, culture supernatant fluid from a *P. multocida*-*M. haemolytica* coculture was also used in this assay, but there was no inhibition of growth of *M. haemolytica*. Direct plating of *P. multocida* on *M. haemolytica* plates yielded the same results. Taken together, these results suggest that inhibition of growth of *M. haemolytica* by *P. multocida* is not me-

diated by a soluble bactericidal compound unless it is very labile or it is present at exceedingly low concentrations.

DISCUSSION

M. haemolytica, *B. trehalosi*, *P. multocida*, and *M. ovipneumoniae* have been identified as pathogens associated with pneumonia in BHS (5, 9, 20, 23, 27). Of these, *M. haemolytica* has been shown to consistently induce fatal pneumonia in BHS under experimental conditions (12, 19, 29). However, *M. haemolytica* has been isolated by culture much less frequently than the others (31, 33). We hypothesized that the growth of *M. haemolytica* is inhibited by other bacteria in the lungs. In an earlier study, Dassanayake et al. (13) found that *B. trehalosi* inhibits the growth of *M. haemolytica* *in vitro*. In the present study we determined whether *P. multocida* inhibits the growth of *M. haemolytica*.

In monocultures, *P. multocida* and *M. haemolytica* exhibited similar growth characteristics as evidenced by overlapping growth curves, and this did not change when strains were resistant to selective antibiotics (Fig. 1). Based on the results of coculture experiments, it is clear that *P. multocida* inhibits the growth of *M. haemolytica* (Fig. 2 and 3) and that close proximity is required for the inhibition (Fig. 5). Furthermore, this phenotype was evident in experiments with several strains (Table 1), indicating that the susceptibility to inhibition is not unique to a particular serotype of *M. haemolytica* and that the inhibitory phenotype is not unique to a particular isolate of *P. multocida*. In all of the coculture experiments, the CFU counts of *M. haemolytica* and *P. multocida* were determined by plating an aliquot of the culture on ampicillin- or rifampin-containing agar plates, respectively. A subset of the colonies growing on the ampicillin and rifampin plates was confirmed to be *M. haemolytica* and *P. multocida*, respectively, by species-specific PCR assays (data not shown).

B. trehalosi inhibits the growth of *M. haemolytica* by a proximity-dependent mechanism (13). *P. multocida* and *B. trehalosi* are closely related bacterial species within the family Pasteurellaceae. Therefore, we tested the hypothesis that *P. multocida* also utilizes a contact- or proximity-dependent inhibition mechanism. Inhibition of growth of *M. haemolytica* by *P. multocida* when the two bacteria were separated by a membrane with a pore size of 8.0 µm (which allows the passage of bacteria), but not when they were separated by a membrane with a pore size of 0.4 µm (which allows the passage of secreted proteins and bacteriophages, but not bacteria) is consistent with a contact- or proximity-dependent mechanism. Certain strains of uropathogenic *Escherichia coli* (EC93) have been shown to inhibit the growth of other strains of genetically different *E. coli* (*E. coli* K-12) by a contact-dependent mechanism (2). Specific ligand interaction between CdiAB from the inhibitory strains and BamA (YaeT) on the surface membranes of target strains mediates the inhibition (3). A similar mechanism may be involved in the inhibition of *M. haemolytica* by *P. multocida*. This speculation is supported by the observation that a potential BamA homologue has been annotated for the *Pasteurella* species (26). Further experiments are necessary to determine whether the mechanism of inhibition by *P. multocida* is similar to that of *E. coli* EC93. *E. coli* 25 and *E. coli* 264 also inhibit several other strains of *E. coli*, including *E. coli* O157:H7 (32). The mechanism of inhibition is proximity dependent, but the molecular basis for the inhibition has not been described in the literature.

Several bacteriophages have been isolated from members of the genera *Pasteurella* and *Mannheimia*, and some of these phages

form plaques with indicator strains of the same species (1, 15). Sawant et al. (32) have shown that the 0.4- μ m-pore-size filters used in the present study are permissive to the diffusion of phages. The lack of inhibition of growth of *M. haemolytica* by *P. multocida* when they were separated by a membrane with a pore size of 0.4 μ m is consistent with the absence of bacteriophage in the culture supernatant fluid. Furthermore, certain bacterial strains of *E. coli* and *Klebsiella* have been shown to produce bactericidal compounds that inhibit competitors in the vicinity (11, 16, 21). We could not demonstrate inhibition of growth of *M. haemolytica* on agar plates using the culture supernatant fluid from *P. multocida* monoculture or *P. multocida* and *M. haemolytica* coculture. Neither the addition of concentrated proteins from the culture supernatant fluid nor direct plating of *P. multocida* caused inhibition of growth of *M. haemolytica*, indicating that an insufficient concentration of any bactericidal compounds, if any, could be detected by our assay. Collectively, these results indicate that the inhibition of growth of *M. haemolytica* by *P. multocida* is mediated by a contact- or proximity-dependent mechanism. It is likely that the contact- or proximity-dependent mechanism involves bactericidal compounds or quorum-sensing molecules that are needed only at very low concentrations to achieve inhibition. The possibility of the “inhibitory molecule” being secreted directly into the target cells cannot be discounted.

B. trehalosi inhibits the growth of *M. haemolytica* (13). *B. trehalosi* grows faster than *M. haemolytica* and achieves a higher final cell density when grown in monocultures. The CFU count for *B. trehalosi* at 24 h is 3 logs higher than that for *M. haemolytica*. However, the growth rate of *P. multocida* in monoculture is comparable to that of *M. haemolytica*, and *P. multocida* very effectively inhibits the growth of *M. haemolytica*. It is tempting to speculate that the mechanism of inhibition of *M. haemolytica* by *P. multocida* is more potent than that of *B. trehalosi*. However, further studies are necessary to confirm this notion. In summary, our study clearly demonstrates that *P. multocida* inhibits the growth of *M. haemolytica*. The inhibition is mediated by a contact- or proximity-dependent mechanism.

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