Role of *Brevundimonas vesicularis* in supporting the growth of *Legionella* in nutrient-poor environments

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SUMMARY

In 1986, we encountered the first case of *Legionella micdadei* pneumonia in Japan. In the follow-up study to determine the infection route of *L. micdadei*, we isolated *Brevundimonas vesicularis* from the shower hose of the patient's home. This motivated us to explore the symbiosis between *B. vesicularis* and *Legionella* in this study. *B. vesicularis* type strain, *B. vesicularis* Kobe strain, *Legionella pneumophila* serogroup 1 type strain, and *L. micdadei* Kobe strain were used. *B. vesicularis* was inoculated into 0.01 M phosphate buffer solution containing artificial sand, and varying concentrations of glucose at 0.1%, 0.01%, and 0.001%. *Legionella* was added to the cultures after ten days of incubation, and *Legionella* viable counts were monitored over time. After three days of incubation, *Legionella* counts increased approximately twofold in flasks containing 0.001% glucose, but *Legionella* counts decreased in both *B. vesicularis* inoculated and non-inoculated flasks containing higher concentrations of glucose. The counts were significantly higher in flasks inoculated with *B. vesicularis* than in non-inoculated flasks throughout the experiments. Under the nutrient-poor conditions, the presence of *B. vesicularis* was found to aid a further increase in *Legionella* counts. Further research is necessary to understand the symbiotic conditions most supporting the growth of *L. micdadei.*

KEY WORDS: Brevundimonas vesicularis, Legionella micdadei, Legionella pneumophila, Symbiosis.

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INTRODUCTION

Legionella is a planktonic bacterium found within protozoa and biofilms in a variety of natural and manmade water systems (Wadowsky *et al*., 1988; Marrào *et al*., 1993; Manpel *et al*., 2006; Piao *et al*., 2006; Söderberg *et al*., 2008). *Legionella* is an endoparasite of living protozoa, with the ability to invade host cells and multiply within them (Rowbotham, 1980). Under illuminated conditions, cyanobacteria and green algae may produce nutrients uesd by *L. pneumophil*a (Tison, 1980; Pope *et al*., 1982; Bohach *et al.*, 1983), but since tap water is shielded from light inside plumbing systems, it has been hypothesized that the growth of *L. pneumophila* is supported by other microorganisms in such environment (Wadowsky *et al*., 1988).

In 1986, we encountered the first case of *Legionella micdadei* pneumonia in Japan (Koide *et al*., 1988). Interestingly, we could not isolate *L. micdadei* from the patient's surrounding environment, despite successful isolation of *L. micdadei* from the environment in other situations (hospital drinking water, hospital shower, biofilm in a hydrothermal area, etc.) (Brown *et al*., 1982; Stout *et al*., 1982; Marrào *et al*., 1993). In a follow-up investigation to identify infection route, source, and reservoir of *L. micdadei*,

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we isolated *Brevundimonas vesicularis* from the shower hose of the patient's home (Koide *et al*.,1989). *B. vesicularis* was isolated from the swab of the rubber shower hose, but it was not isolated from the storage water tank, shower water, or a swab of the shower head (Koide *et al*., 1989). This finding suggested that the isolate might be an extreme biofilm-forming bacterium, as opposed to a free-floating bacterium. At the patient's home, municipal tap water was stored in a plastic tank in his terraced garden to be used for drinking and shower water. The patient generally showered daily suggesting that the shower system might be a reservoir for bacteria.

Previous research has reported the symbiosis between heterotrophic bacteria and *Legionella*, but these studies were not able to detect the specific species of bacteria (Stout *et al*., 1985; Wadowsky *et al*., 1985; Guerrieri *et al*., 2007). Therefore, we investigated the relationship between *B. vesicularis* and *Legionella* in a laboratory setting to determine whether *B. vesicularis* can support the growth of *Legionella*. Since *B. vesicularis* utilizes glucose and *Legionella* does not, we also investigated the influence of varying glucose concentrations on this relationship.

MATERIALS AND METHODS

Bacterial strains

The following four strains were used in this study: *B. vesicularis* type strain ATCC11426, *B. vesicularis* Kobe 1005 strain (isolated from the shower hose swab in the patient's residence in 1987), *L. pneumophila* serogroup 1 type strain ATCC33152 Philadelphia-1, and *L. micdadei* Kobe 86-009 strain (isolated from the lungs of the *L. micdadei* pneumonia patient during the autopsy in 1986).

Determination of experimental conditions

We grew cultures in 200-mL flasks containing 100 mL of 0.01 M phosphate buffer solution (PBS, Na_2HPO_4 - KH_2PO_4, pH 7.0), and 1 g of artificial sand (Chamotte, 1.0-1.2 mm diameter, Zeolite Inc., Fukuoka, Japan). Artificial sand was washed ≥5 times with 200 mL of distilled water before use. Various concentrations of *B. vesicularis* and glucose (Wako Pure Chemical Industries Inc., Osaka, Japan) were added. Glucose was added at concentrations of 0.1% (0.1 g/100 mL), 0.01% (0.01 g/100 mL), and 0.001% (0.001 g/100 mL).

B. vesicularis type strain or *B. vesicularis* Kobe 1005 strain were inoculated onto a *Legionella* agar base (BCYEα agar without L-cysteine, ferric pyrophosphate; adjusted to pH 6.9 with 1N KOH; Becton, Dickinson and Company, Sparks, MD, USA). The plate was incubated for 2-3 days at 30°C. Colonies were harvested in distilled water, and concentrations was adjusted to approximately 10^8 colony forming units (cfu) /mL using a Vitek Colorimeter (BioMerieux Vitek Inc., Hazelwood, MO, USA). One hundred µL of the dilution of this solution were added to the flasks. Flasks were incubated at 30°C. After ten days of incubation, viable counts of *B. vesicularis* and glucose concentration were evaluated at various time points. Glucose concentration was measured using the hexokinase method. To determine the count of *B. vesicularis,* 100 µL of media were serially diluted tenfold, poured onto *Legionella* agar base, and incubated at 30°C for five to seven days. The number of colonies on the agar plates was counted, and the bacterial numbers were determined from these counts. Experiments were conducted twice using different concentrations of inoculum (10^3 and 10^5 cfu/mL).

Symbiosis experimental design

L. pneumophila type strain ATCC33152 or *L. micdadei* Kobe 86-009 strain was inoculated onto BCYEα agar (pH 6.9, Becton, Dickinson and Company), and incubated for three to five days at 37°C. Colonies were harvested in distilled water, and the concentrations were adjusted to approximately $3x10^7$ cfu/mL. This solution was diluted 1:100, and 100 µL were added to a flask containing *B. vesicularis* (10^4 cfu/mL inoculation numbers) that had been incubated for ten days.

At various time points during the 30°C post-incubation, viable counts of *Legionella* were evaluated. To determine the count of *Legionella,* 100 µL of the media were serially diluted tenfold, plated on BCYEα agar, and incubated at 40°C for two days and then at 37°C for eight to 12 days in humidified air. The initial incubation at 40°C was necessary to prevent the growth and

colony formation of *B. vesicularis*. Experiments were conducted in triplicate, and means were calculated for comparison.

RESULTS

Determination of experimental conditions

The *B. vesicularis* type strain was inoculated in flasks at concentrations of 10^3 or 10^5 cfu/mL. In the flask containing 0.001% glucose, the *B. vesicularis* count remained stable (10^4-10^5 cfu/mL) throughout the experiment. In the flask containing 0.01% glucose, the *B. vesicularis* count decreased gradually, but maintained a level of 10^2-10^3 cfu/mL at four weeks post inoculation. In the flask containing 0.1% glucose, the *B. vesicularis* population decreased gradually and disappeared at three to four weeks post-inoculation. By day 10, the *B. vesicularis* in the flask containing no glucose had died. Glucose concentration remained stable throughout the experiments. Replicate experiments produced almost identical results regardless of inoculum numbers.

In addition, the *B. vesicularis* Kobe 1005 strain was inoculated in flasks at concentrations of 10^3 or 10^5 cfu/mL. In the flasks containing 0.001% glucose and 0.01% glucose, the *B. vesicularis* count remained stable (10^5-10^6 cfu/mL) throughout the experiment. In the flask containing 0.1% glucose, the *B. vesicularis* count maintained a level of 10^4-10^5 cfu/mL for four weeks. The *B. vesicularis* Kobe 1005 strain was able to survive at 10^4-10^5 cfu/mL in flasks containing no glucose for the duration of the experiment. Glucose concentration remained stable throughout the experiments. Replicate experiments produced almost identical results regardless of inoculum numbers.

Thus, we used approximately 10^4 cfu/mL inoculum number of *B. vesicularis* for the following symbiosis experiments.

Symbiosis experiment

Symbiosis between L. pneumophila type strain ATCC33152 and B. vesicularis type strain ATCC11426

At lower levels of glucose (0.01% and 0.001%), *Legionella* (*L. pneumophila* type strain ATCC33152) survival was enhanced by the addition of *B. vesicularis* type strain ATCC11426 (*Fig. 1*). In fact, *Legionella* counts actually increased in *B. vesicularis*-inoculated flasks at the lowest level of glucose (0.001%), whereas they decreased in identical flasks that were not *B. vesicularis*-inoculated. At the highest level of glucose (0.1%), *Legionella* counts were non-existent by three days post inoculation, the only surviving *Legionella* was in *B. vesicularis*-inoculated flasks (containing 0.001% glucose) (Figure 1).

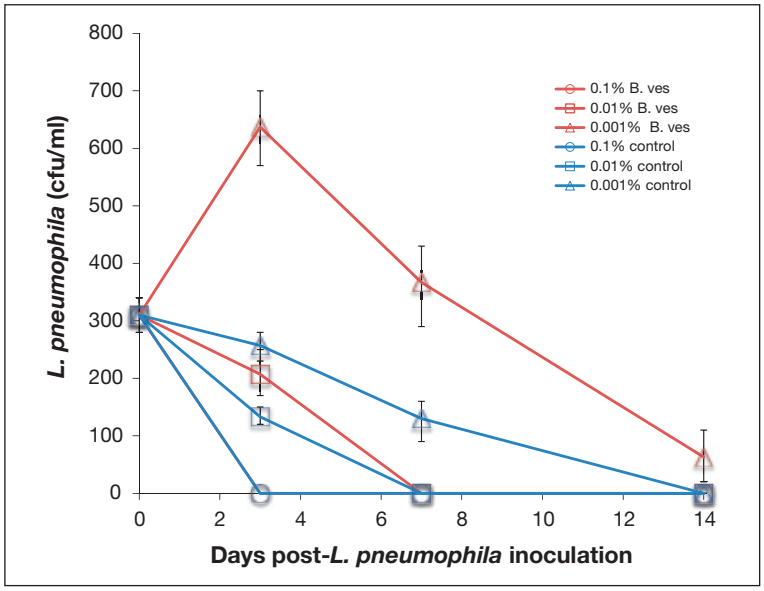

FIGURE 1 - *Mean viable counts of L. pneumophila type strain ATCC33152 at varying glucose concentrations (0.1%, 0.01%, and 0.001%) in B. vesicularis type strain ATCC11426-inoculated media. Vertical bars indicate the range of L. pneumophila counts of 3 independent experiments.*

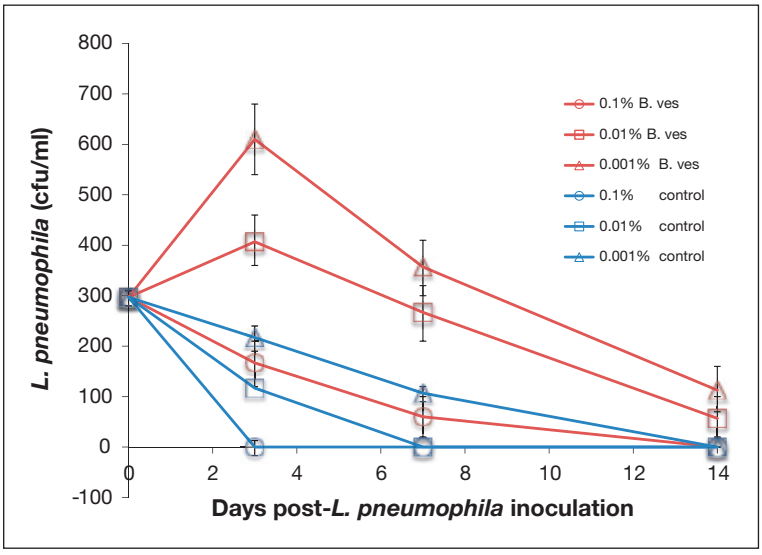

FIGURE 2 - *Mean viable counts of L. pneumophila type strain ATCC33152 at varying glucose concentrations (0.1%, 0.01%, and 0.001%) in B. vesicularis Kobe 1005 strain-inoculated media. Vertical bar indicate the range of L. pneumophila counts of 3 independent experiments.*

Symbiosis between L. pneumophila type strain ATCC33152 and B. vesicularis Kobe 1005 strain

At all levels of glucose (0.1%, 0.01%. 0.001%), *Legionella* (*L. pneumophila* type strain ATCC33152) survival was enhanced in the presence of *B. vesicularis* Kobe 1005 strain (*Fig. 2*). However, independent of *B. vesicularis* Kobe strain inoculation, *Legionella* survived better at lower concentrations of glucose. By 14 days post inoculation, the only surviving *Legionella* was in flasks containing *B. vesicularis* Kobe strain plus a low (0.001%) or moderate (0.01%) concentration of glucose (Figure 2).

Symbiosis between L. micdadei Kobe 86-009 strain and B. vesicularis Kobe 1005 strain

At lower levels of glucose (0.001% and 0.01%), *L. micdadei* Kobe 86-009 strain survival was enhanced in the presence of *B. vesicularis* Kobe 1005 strain (Figure 3). *Legionella* counts actually increased after three days at 0.001% and 0.01% glucose in the presence of *B. vesicularis*. By 14 days post inoculation, the only surviving

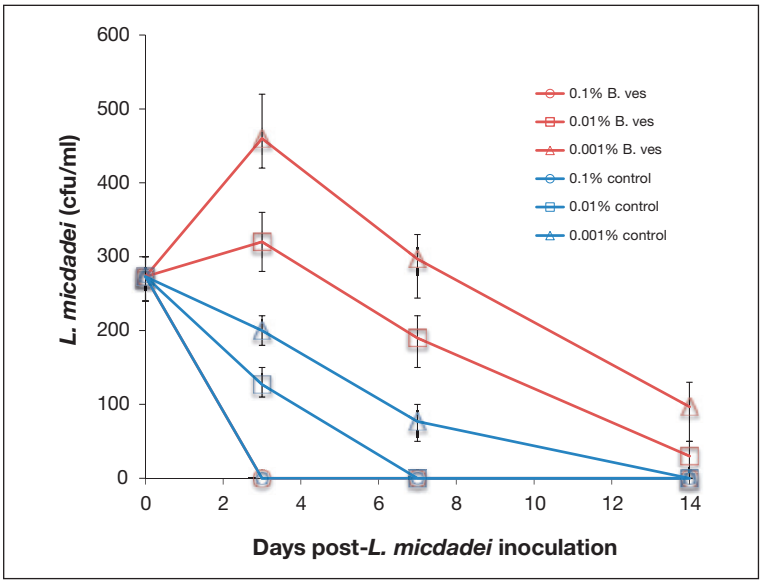

FIGURE 3 - *Mean viable counts of L. micdadei Kobe 86-009 strain at varying glucose concentrations (0.1%, 0.01%, and 0.001%) in B. vesicularis Kobe 1005 strain-inoculated media. Vertical bar indicate the range of L. micdadei counts of 3 independent experiments.*

Legionella was in *B. vesicularis*-inoculated flasks (containing 0.001% or 0.01% glucose) (Figure 3). Overall, *L. micdadei* showed decreased survival in comparison to *L. pneumophila*, although the growth trends were the same.

DISCUSSION

In 1953, Büsing *et al.* first reported *B. vesicularis* as the isolate from the urinary bladder epithelium of the leech *Hirudo medicinalis* (Büsing *et al*., 1953).

They designated this bacterium *Corynebacterium vesiculare*. In 1964, Galarneault and Leifson classified this bacterium as *Pseudomonas vesicular* (Ballard *et al*., 1968; Galarneault *et al.*, 1964;). Later in 1994, following the designated name *Pseudomonas vesicularis*, Segers *et al.* proposed the new genus *Brevundimonas* against *Pseudomonas vesicularis* and *Pseudomonas diminuta* (Segers *et al*., 1994). At present, the genus *Brevundimonas* consists of 17 identified species (Estrela *et al*., 2010). In our case, we identified the isolate as *Pseudomonas vesicularis* at that time in 1987. We then performed 16S-rDNA sequencing. This isolate formed a coherent cluster with *B. vesicularis, Brevundimonas nasdae, Brevundimonas intermedia,* and *Brevundimonas aurantiaca,* as mentioned by Li *et al*, 2004. This isolate phenotypically resembled *B. nasdae*, but was more similar to *B. vesicularis* on the basis of 16S-rDNA sequencing.

According to the few reports concerning *B. vesicularis*, this bacterium has been isolated from environments such as aqueous reservoirs, shower hoses, and leeches, but it is rarely isolated from human clinical specimens (Aspinall *et al*., 1989; Planes *et al*., 1992; Davis *et al*., 1997; Li *et al*., 2004; Lee *et al*., 2011).

Furthermore, even fewer reports have been published regarding the symbiosis between bacteria and *Legionella*. Wadowsky *et al.* reported that one strain of *Flavobacterium breve* supports satellite growth of *L. pneumophila* on an L-cysteine-deficient medium (Wadowsky *et al*., 1983). The same group also reported the multiplication of *L. pneumophila* in tap water infected with the naturally occurring suspension from a gymnasium hot water tank at an increasing level of 1.3 log CFU (Wadowsky *et al*., 1985). A mixed suspension of four kinds of unidentified non-*Legionellaceae* bacteria isolated from this hot water tank slightly increased the *L. pneumophila* concentration at 0.2 to 0.4 log CFU level (Wadowsky *et al.*, 1985). This group speculated that there are bacteria present in plumbing systems that may supply *L. pneumophila* with at least some of the amino acids required for the metabolism of *L. pneumophila*, but later they also mentioned that these bacteria alone fail to support the multiplication of *L. pneumophila* in tap water (Wadowsky *et al*, 1988). These findings, taken together with our discovery of *B. vesicularis* in the home of a patient infected with *L. micdadaei*, led us to explore the ability of *B. vesicularis* to support the growth of *Legionella*.

Prior to conducting these symbiotic experiments in the laboratory,we first needed to establish growth conditions to support *B. vesicularis* growth. Using replicate experiments, we determined the necessary inoculum number of *B. vesicularis* to be 10^4 cfu/mL.

Once these conditions were established, we tested the symbiosis between *Legionella* and *B. vesicularis* by first inoculating flasks with *B. vesicularis* (type strain or Kobe strain), then with *Legionella* (*L. pneumophila* serogroup 1 type strain or *L. micdadei* Kobe strain). The survival of both *Legionella* strains was enhanced in the presence of *B. vesicularis* (Figures 1, 2, 3). This enhancement was observed under low glucose concentrations.

Glucose concentration did not aid the growth of *B vesicularis*. *B vesicularis* grew better at low glucose concentrations than at high glucose concentrations. Therefore, the growth of *Legionella* was linked to the number of *B vesicularis* present in the culture medium.

Manpel *et al.* reported that *L. pneumophila* did not replicate in a defined biofilm under nutrient-poor conditions (Manpel *et al*., 2006), but our results contradict this finding. In fact, our data suggest that in the presence of trace amounts of nutrients, *B. vesicularis* could potentially support the growth of *Legionella*. Manpel *et al.* also reported that some bacteria (*Corynebacterium glutamicum*, *Klebsiella pneumonia, Pseudomonas* spp.) can exert a negative effect on the growth of *Legionella*. It will be in-

teresting to perform the experiment using these bacteria as the negative control.

In order to understand the mechanism that allows for the symbiotic growth of *L. micdadei* and *L. pneumophila* in the presence of *B. vesicularis*, we must further investigate the symbiotic conditions, including:

1) nutrients used by *B. vesicularis* such as L-proline, L-aspartate, L-serine, D-alanine, succinate, and fumarate, alone or in combination with glucose;
2) the timing of *Legionella* and *B. vesicularis* co-inoculation, e.g. the simultaneous inoculation of *Legionella* and *B. vesicularis* to the flask, or preceded *Legionella* inoculation prior to *B. vesicularis* inoculation;
3) incubation temperature (20-37°C).

In conclusion, in a nutrient-rich environment, amoebae may act as symbiotic partners of *Legionella*. In an illuminated environment, blue-green algae mainly act or co-act with amoebae as symbiotes of *Legionella*. However, in dark and very nutrient-poor environments, *B. vesicularis* may potentially support the cryptic growth of *Legionella*. More work is necessary to understand the factors that allow for this symbiotic growth. Our research also suggests that there may be other bacteria with the potential to support *Legionella* in low nutrient environments.

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