

Influence of aquatic microorganisms on *Legionella pneumophila* survival

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SUMMARY

The ability of aquatic bacteria *Pseudomonas fluorescens* SSD (Ps-D) and *Pseudomonas putida* SSC (Ps-C) to support the persistence of *Legionella pneumophila* (Lp-1) in an artificial water microcosm was investigated for 42 days, at two different incubation temperatures. At 4 °C, individually suspended Lp-1 was no longer detectable just after 24 hours, while in co-cultures with *Pseudomonas*, Lp1 showed a better survival capability. At 30 °C, Lp-1 alone displayed high survival rates over the entire period of observation. When Lp-1 was inoculated with Ps-C and Ps-D, its count showed a marked decrease, followed by a gradual and constant decline.

KEY WORDS: *Legionella pneumophila*, *Pseudomonas*, Survival, Aquatic habitats

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A number of studies are available on the ability of *Legionella pneumophila* to survive in aquatic systems and the role of amoebal hosts in its ecology is widely documented (Borella *et al.*, 2005). Nevertheless, few studies are reported to date on the relationship between *Legionella* and other water-borne microorganisms, frequently isolated from the same samples (Sommerse *et al.*, 1996). The microbial flora of aquatic environments is generally made up of several species of bacteria, fungi and protozoa. Within this complex community, a large number of interactions occur, in particular among different bacteria (Messi *et al.*, 2003; Messi *et al.*, 2005). Thus, we cannot exclude that legionella survival may also be positively or negatively influenced by other aquatic germs. A mutualistic mechanism was observed in complex media lack-

ing cysteine or iron salts, unable to support the growth of *L. pneumophila*, where the bacterium formed satellite colonies around strains of some common aquatic species, including *Flavobacterium*, *Pseudomonas*, *Alcaligenes* and *Acinetobacter* (Wadowsky *et al.*, 1985). A role of aquatic bacteria in supporting the growth or influencing the survival of *L. pneumophila* in simple media or water has been supposed. In a study on cooling towers, the highest counts of legionellae were observed in samples with high concentrations of heterotrophic bacteria, even if the correlation between the occurrence of the pathogen and heterotrophic bacteria was not significant (Yamamoto *et al.*, 1992). In this case the authors suggested an indirect or synergistic relationship, rather than a direct relationship with specific bacteria. Wadowsky *et al.* (1991) observed that killed cells of *Pseudomonas paucimobilis* represented a source of nutrients for host protozoa, thus playing a positive role in legionella survival. Surman *et al.* (1994) demonstrated that bacteria isolated from biofilms grown in a continuous culture model system enhanced the viability of *L. pneumophila* when inoculated onto

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R2A medium, a phenomenon not observed when heat-killed and cell-free extracts were used. Lee and West (1991) confirmed that other bacteria could represent an essential nutrient for the protozoa, although the ability of these microorganisms to support the growth of legionellas under natural conditions is not proven.

In this study, the survival capability of a strain of *L. pneumophila* was investigated in an artificial water microcosm, in the presence of other water-borne bacteria to better define their ability to support/inhibit the environmental persistence of the pathogen. Two strains belonging to the genus *Pseudomonas*, the most frequently present in water habitat, were chosen as representative of the natural microflora of aquatic environments.

L. pneumophila serogroup 1 strain (Lp-1), isolated from hot tap water and already employed in previous studies (Guerrieri *et al.*, 2005), was subcultured on buffered charcoal-yeast extract agar supplemented with α -ketoglutarate (aBCYE, OXOID Milan, Italy) (Edelstein, 1981) and incubated for 4 d at 36 °C in a moist chamber with 2.5% CO₂ added.

Pseudomonas fluorescens SSD (Ps-D) and *Pseudomonas putida* SSC (Ps-C), isolated in our laboratory from mineral water samples (Messi *et al.*, 2002), were used as aquatic bacteria. The isolates were recovered by using standard procedures for screening of coliforms in water and wastewater (APHA, 1995), and were tested for oxidase production (*NN*-dimethyl-*p*-phenylenediamine dihydrochloride, Sigma Chemical Co., St. Louis, MO, USA). Identification was made according to standardized tests API 20 NE strips (bioMérieux, Marcy l'Etoile, France). Cultures of *Pseudomonas* were then grown in Tryptic Soy Broth (TSB, Difco Laboratories, Detroit, MI) supplemented with 0.6% yeast extract (TSB-YE) (Difco) at 30 °C for 18 h.

All the strains were stored in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 2.9 g Na₂HPO₄·12H₂O, 0.2 g KH₂PO₄ with 1 l of distilled water) supplemented with 30% (vol./vol.) glycerine at -80 °C.

Cell suspensions of Lp-1 were obtained by collecting colonies, grown on aBCYE agar plates, with a sterile swab and suspending the same in 5 ml of sterile deionized water. For *Pseudomonas* overnight cultures grown in TSB-YE at 30 °C for

18 h were used. All cultures were centrifuged at 2,000 x g for 20 min. After discarding the supernatant fluid, the pellets were re-suspended in 5 ml sterile deionized water. Centrifugation, supernatant discard and re-suspension were repeated three times. The density of the final suspensions was measured by the plate count method. The survival experiments were carried out by the previously described "macromethod" (Guerrieri *et al.*, 2005) on samples of tap water filtered through membrane filters (0.45-mm-pore-size Sartorius AG, Goettingen, Germany) to remove autochthonous microbiota. Eight hundred ml-volumes of the water were added to sterile Pyrex glass flasks (1 l), cleaned with acid and autoclaved before use.

The flasks were then inoculated with 0.8 ml of the test strain suspensions in different combinations (Lp-1 alone, Lp-1 with Ps-D, Lp-1 with Ps-C, Ps-D alone and Ps-C alone) to yield a final bacterial count of 4 x 10⁶ cfu ml⁻¹ for Lp-1, 3 x 10⁵ cfu ml⁻¹ for Ps-D and 5 x 10⁵ cfu ml⁻¹ for Ps-C. The flasks were incubated for 42 days at 4 °C and 30 °C.

At regular intervals, daily during the first week and every three days afterwards, the bacterial count was determined by spreading 0.1 ml of serial tenfold dilution samples on appropriate agar plates. The medium α -BCYE was used for singly inoculated legionella counts, while the more selective MWY and GVPC (OXOID Milan, Italy) were used to enumerate legionella cells in the presence of other bacteria; Tryptic Soy Agar (TSA, Difco Laboratories, Detroit, MI) was used for the other bacterial counts.

Plates were incubated at 36 °C in a moist chamber with 2.5% CO₂ added for Lp-1 and at 30 °C for pseudomonas. During incubation, plates were examined daily for up to 7 d, and finally the grown colonies counted. The experiment was conducted in triplicate and the bacterial count was performed on three plates. The arithmetical mean of three sample determinations, expressed as log bacterial count, was plotted against incubation time (days).

The results obtained at 4 °C and 30 °C are shown in Figures 1 and 2. Reported data refer to the experiments performed with the Ps-D strain, as the results obtained employing Ps-C were similar. At 4 °C (Figure 1), individually suspended Lp-1 was no longer detectable after just 24 hours.

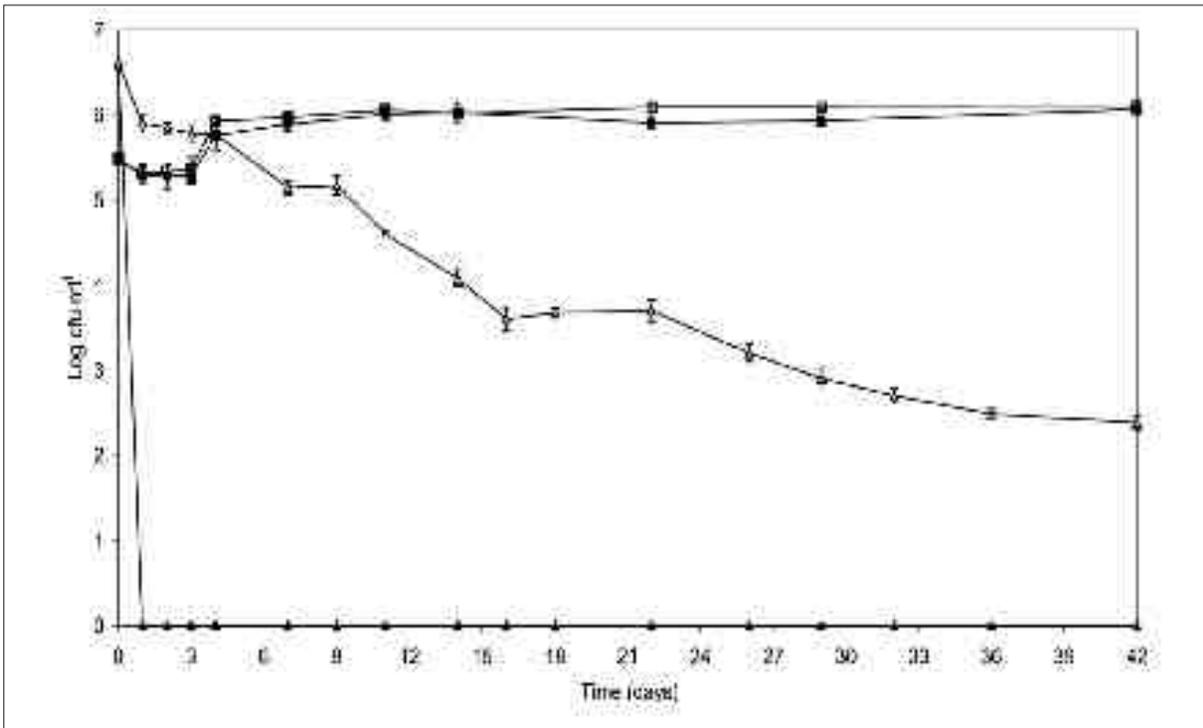


FIGURE 1 - Viable cell count of *Legionella pneumophila* serogroup 1 (*Lp-1*) and *Pseudomonas fluorescens* SSD (*Ps-D*) at 4°C. ▲ *Lp-1* alone; △ *Lp-1* in co-culture with *Ps-D*; ■ *Ps-D* alone; □ *Ps-D* in co-culture with *Lp-1*.

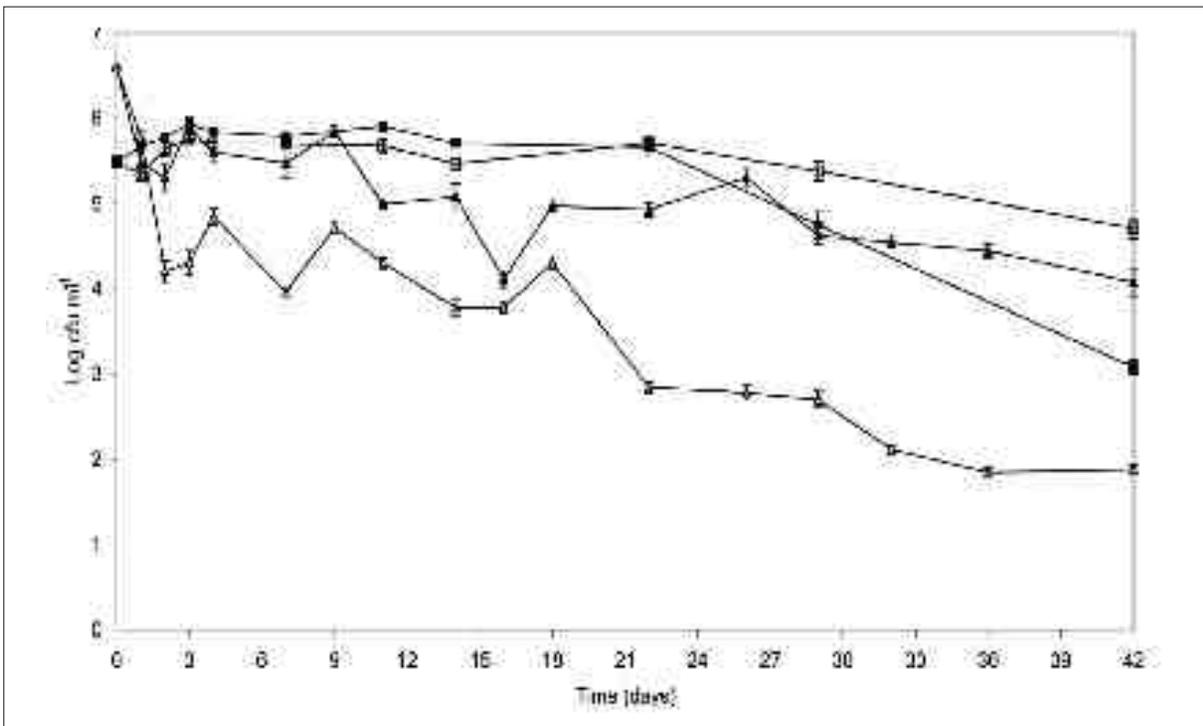


FIGURE 2 - Viable cell count of *Legionella pneumophila* serogroup 1 (*Lp-1*) and *Pseudomonas fluorescens* SSD (*Ps-D*) at 30°C. ▲ *Lp-1* alone; △ *Lp-1* in co-culture with *Ps-D*; □ *Ps-D* alone; ■ *Ps-D* in co-culture with *Lp-1*.

Lp1, when inoculated with Ps-D, showed an initial decrease (about 0.5 log) and then remained nearly constant until the 4th day of incubation. Successively it showed a progressive decrease, with a final viable count of about 10^2 cfu ml⁻¹ after 42 days of incubation. This trend supports the evidence of a favouring role of *Pseudomonas* on the survival capability of *Legionella* under stressing conditions (very low temperature). We suggest that this positive effect may be an indirect action due to increased organic compounds in the medium. These substances derive from the metabolic activity of *Pseudomonas*, which is able to actively multiply also under low temperature conditions. Indeed, for both the single inoculated and the co-cultured experiments, after an initial decline (2-3 log), the viable count of Ps-D gradually increased and remained nearly constant (about 10^{5-6} cfu ml⁻¹) until the 42th day. The good survival capability of *Pseudomonas* strains in artificial water microcosms has been previously demonstrated (Messi *et al.*, 2002) and the present investigation confirms that this happens also under unfavourable physical conditions.

In the experiments carried out at 30 °C (Figure 2), after the initial physiological decrease, single inoculated Lp-1 showed high survival rates over the entire period of observation (10^4 cfu ml⁻¹ after 42 days). In this case Ps-D displayed a negative influence on the survival capability of Lp1, which showed a marked initial decrease, if compared to the control, followed by a gradual and constant decline. However, *Legionella* was still recovered with a count of c.a. 10^2 cfu ml⁻¹ at the end of the experiment. In this case a competition for nutrients, resulting more favourable for *Pseudomonas*, may be suggested. With regard to Ps-D, the viable count of both the single inoculated and the co-cultured strain, after a physiological initial decrease, remained nearly constant until the 21st day. Thereafter Ps-D alone decreased more than Ps-D in co-culture.

The results of the present study suggest that the bacteria belonging to autochthonous microflora of the aquatic habitat may directly influence *L. pneumophila* and therefore play a role in its ecology, depending on the environmental conditions. Actually, the survival capability of the pathogen was enhanced by the presence of *Pseudomonas* strains at a temperature of 4 °C, while it resulted negatively influenced at a growing tempera-

ture of 30 °C. This last observation could be in accordance with Leoni *et al.* (2001) who reported a statistically significant inverse correlation between legionellae and Gram negative bacteria in samples from the showers of several swimming pool establishments. The water temperature was higher than that employed in this study, but in any case this phenomenon was observed only when the temperature was under 43 °C and other competitor microorganisms were not present. Obviously, our experiments cannot completely reproduce the real conditions of natural habitats, characterized by a more complex community in which both the presence of protozoa and the biofilm formation represent essential features in *Legionella* ecology (Borella *et al.*, 2005). As many aquatic bacteria, including *Pseudomonas*, are able to survive and multiply within protozoa or may act as intracellular parasites (Inglis *et al.*, 2000; Winięcka-Krusnell and Linder, 2001), a competition for the protozoan host may exist under natural conditions. This aspect deserves special consideration also because the capability of intracellular life is considered an important virulence factor, besides other traits commonly shown by aquatic bacteria (Bondi *et al.*, 2000).

Furthermore, the interactions which occur within biofilms between *L. pneumophila* and aquatic bacteria have recently been studied. In biofilms formed under artificial conditions, the presence of bacteria like *Empedobacter breve* or *Micobacterium* spp. favoured the adherence and persistence of *L. pneumophila*. On the contrary, *Pseudomonas* spp., *Corynebacterium glutamicum* and *Klebsiella pneumoniae* reduced the adherence of *Legionella* and accelerated its detachment from the biofilm (Mampel *et al.*, 2006). Based on these observations, the authors suggest the important role of the other bacteria in modulating the behaviour of *L. pneumophila*. As *Pseudomonas* represents the major genus involved in biofilm formation in aquatic environments (Donlan, 2002), studies are in progress to investigate the interactions between *Pseudomonas* or *Pseudomonas*-like bacteria and *L. pneumophila* occurring in biofilms.

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