Long-Term Starvation-Survival of *Erwinia amylovora* in Sterile Irrigation Water

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Abstract

The role of irrigation water in disseminating *Erwinia amylovora* is not fully recognized, and the survival of the bacterium in natural water has not been carefully investigated. This risk has been underestimated, since it is generally considered that *E. amylovora* survives only for a short period in water and its isolation from natural water samples has not been reported. The main goal of this study has been to clarify whether *E. amylovora* could survive under nutrient starvation conditions usually found in aquatic environments and if it is nonculturable on solid media when recovered from sterile natural water. Infectivity of *E. amylovora* cells in water was evaluated by using immature pear fruits. Total and viable cell counts were monitored by the Live/Dead viability kit, and culturability by plate counts on King's B medium. *E. amylovora* was able to survive in water from different sources showing a long persistence in irrigation water and maintaining its infectivity for green pears. However, a progressive loss of culturability on solid media by 2 to 3 logarithmic units during storage time was observed, the rate at which cells became nonculturable dependent on the type of water used. A significant difference in the time to nonculturability between cells maintained in irrigation water and those kept in deionized water was observed, associated with the content of dissolved compounds, which differ for these two types of water. Since bacterial cells maintained their viability, it seems that the oligotrophic conditions found in natural aquatic environments could allow the survival of the fireblight pathogen. Further, the maintenance of the pathogenicity of *E. amylovora* supports the possible role of water as a reservoir and vehicle for transmission of this pathogen.

INTRODUCTION

Rain and irrigation water have been reported to be involved in fire blight transmission at short distances (McManus and Jones, 1994; Thomson, 2000). However, the role of water in the dissemination of this bacterium is not fully recognized. This threat has been overlooked because of the general consideration that this pathogen survives only for short periods in distilled water (Goodman, 1983) and no reports are available on its isolation from natural water samples.

Despite the numerous studies with *Erwinia amylovora*, the information about its hidden life outside host plants is still scarce. In fact, the presence of this bacterium in aquatic and soil habitats where its survival could be negatively affected by both biotic and abiotic factors is practically unknown, as well as the importance of these possible reservoirs to spread the pathogen. A major limitation in soil and water ecosystems is the low nutrient availability, and under starvation conditions bacteria may slow down their growth or show a progressive loss of culturability or just die. It is widely recognized that many bacterial species undergo a starvation-survival response under these oligotrophic conditions (Morita, 1997). Other species can survive by entering the viable but nonculturable (VBNC) state, in which the cells are unable to grow on solid media but remain viable (Roszak and Colwell, 1987). Research on phytopathogenic bacteria such as other *Erwinia* spp., *Agrobacterium tumefaciens* and *Ralstonia solanacearum* has shown

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the importance of water and soil habitats as reservoirs and dissemination routes of these pathogens (Cappaert et al., 1988; Scanferlato et al., 1989; Armon et al., 1995; Manahan and Steck, 1997; van Elsas et al., 2000, 2001). Although *E. amylovora* may survive in soil for weeks and the presence of lytic phages from soil beneath blighted trees has also been reported (Thomson, 2000; Hildebrand et al., 2001) nothing is known about the survival of this pathogen in natural water.

The objective of this study has been to clarify whether *E. amylovora* is able to survive under the starvation conditions of aquatic environments, and if it becomes nonculturable on solid media when recovered from microcosms of sterile natural water. Since the overwintering of this bacterium is still poorly understood we have also investigated the possible influence of low temperature on its survival in irrigation water. The pathogenicity of *E. amylovora* cells in water has also been evaluated.

MATERIALS AND METHODS

Bacterial Strains and Water Microcosms

One reference and three Spanish strains of *E. amylovora* from different origins were: CFBP1430 (CFBP, Collection Francaise de Bactéries Phytopathogènes) and from Spain IVIA1509, IVIA1525-6 and IVIA1892-1. For the starvation experiments the microcosms were prepared using four types of water (0.2 μm-filtered and sterilized): deionized water, drinking water, rain water and irrigation water. Microcosms were inoculated with 10^7 cfu/ml of each *E. amylovora* strain, as described before (Biosca et al., 2003) and maintained at 26°C up to six months. To investigate the influence of low temperature in the survival of *E. amylovora* in water, two strains and irrigation water were selected. The microcosms prepared as described above were incubated at 26 and 5°C during three months.

E. amylovora Cell Counts

Culturable, viable and total cell counts from water microcosms were monitored at time zero, after 48 h and weekly, for at least six months. Plate counts were done on King's B medium (King et al., 1954) while total and viable cell counts were determined by the Live/Dead viability kit (Molecular Probes Inc., Eugene, Oregon) using an epifluorescence microscope.

Infectivity Assays in Green Pears

The pathogenicity of starved and low temperature starved cells of *E. amylovora* from each irrigation water microcosms was assayed by inoculation on green pears (cultivar 'Bartlett') as described by López (2004), after three and six months of incubation. *E. amylovora* cells from each strain grown on King's B medium were used as positive controls and sterile PBS as negative control. Appearance of symptoms at 26°C was monitored daily.

RESULTS

Survival of *E. amylovora* in Water

The four strains of *E. amylovora* were able to survive in different types of sterile water under starvation conditions at 26°C for 21 days. The survival of strain CFBP1430 is shown in Fig. 1. A decline in the culturability on King's B solid medium was observed depending on the type of water used. In deionized water a reduction of about 4 logarithmic units was observed in the number of culturable cells after three weeks. In drinking water a dramatic decline in the culturability was observed during the first 24-48 h (from 10^6 to 10^{4-3} cfu/ml) probably due to the presence of residual chlorine, but afterwards the culturable cell numbers were recovered and maintained at about 10^5 cfu/ml. The culturability in rain water and irrigation water was the highest with only a slight decline around 1-2 logarithmic units on day 21. A significant difference in the time

to non-culturability between cells maintained in natural water and those kept in deionized water was observed due to dissolved compounds as the main difference between these two types of water.

The starvation-survival response of *E. amylovora* in irrigation water after six months at 26°C was similar for all the strains, demonstrating its long survival in sterilized water. The starvation-survival response of the French reference strain is represented in Fig. 2. Total and viable bacterial counts remained quite similar to those at the inoculation time, about 10^8 and 10^7 cells/ml, respectively, while culturable counts declined progressively up to 2-3 logarithmic units over the experimental period. The differences observed between the numbers of viable and culturable cells indicate that a fraction (2-3 log units) of the viable population of *E. amylovora* in irrigation water lost its culturability on solid medium.

Regarding experiments in water at 5°C, the number of culturable cells on King's B solid medium remained at the initial levels (approx. 10^7 cfu/ml) during three months, as shown in Fig. 3 for strain CFBP 1430.

Pathogenicity Assays

E. amylovora cells starved for three and six months in irrigation water at 26°C were able to produce typical necrosis and exudates in green pears. Starved cells after three months at 5°C also developed the same symptoms. *E. amylovora* cells were recovered from affected fruits.

DISCUSSION

The role of irrigation water as a source for *E. amylovora* also depends on the survival time of the bacterium in water. Experiments of the present study demonstrate that *E. amylovora* is able to survive in water from different sources including irrigation water. These results are in contrast to the short survival time previously reported for suspensions of this bacterium in distilled water (Goodman, 1993), and are similar to those reported for other important plant pathogenic bacteria, which have been shown to survive for an extended period in water (Manahan et al., 1997; van Elsas et al., 2001; Biosca et al., 2003). However, a progressive loss of growth on solid media (from 2 to 3 log units) was observed, dependent on the nutrient content and the presence of chlorine in different types of water. Similar results have been reported in other bacteria exposed to the starvation conditions characteristic of water ecosystems and/or other stress factors such as chlorine (Byrd et al., 1991; Manahan et al., 1997; Ghezzi and Steck, 1999). The existence of viable but nonculturable (VBNC) cells of *E. amylovora* could lead to an understimation of the pathogen population from environmental sources when based on culture methods. Consequently, the epidemiological significance of this bacterial state on the life of this pathogen outside host plants and the disease cycle should be estimated.

Little is known about the survival of *E. amylovora* in the cold but a negative effect of cold storage in saline solution and apple calyxes has been reported (Hale et al., 1999). In the present study, *E. amylovora* cells kept at 5°C in sterile irrigation water retained their culturability on King's B solid medium at the level of inoculation, while a decline was observed in the microcosms incubated at 26°C within three months. Then, low temperature does not seem to decrease its survival in water and it has been described that this pathogen can grow at 3-5°C (Billing et al., 1961). The former suggests that the overwintering of *E. amylovora* may be more related to the lack of nutrients in the plant during host dormancy (Vanneste and Eden-Green, 2000) than to low temperatures. In fact, nutritional differences have been suggested to explain a higher survival of *E. amylovora* in nutrient broth than in saline solution at 0°C (Hale et al., 1999).

Inoculation assays have shown that *E. amylovora* cells were pathogenic on green pears after being maintained for six months in sterile irrigation water. Such starved cells were also infective when incubated at low temperature for three months. Thus, the maintenance of the pathogenicity of *E. amylovora* in water could support a possible role of water as a reservoir and dissemination route of the bacterium.

In summary, these results have shown that *E. amylovora* is able to survive and remain infective for six months in sterile water, suggesting that the oligotrophic conditions found in natural aquatic environments may allow the survival of the fire blight pathogen. Therefore, the risk of waterborne transmission of *E. amylovora* by irrigation exists, but further studies are in progress to determine the real importance of water in fire blight epidemiology and management.

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Figures

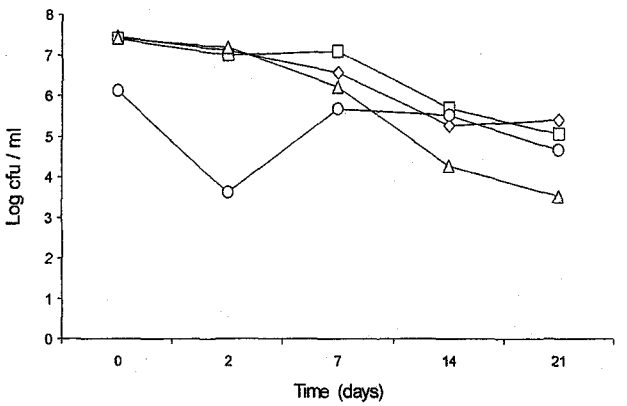

Fig. 1. Culturability of *E. amylovora* in different water environments at 26°C: Δ, deionized water; ○, drinking water; □, rain water and ◇, irrigation water.

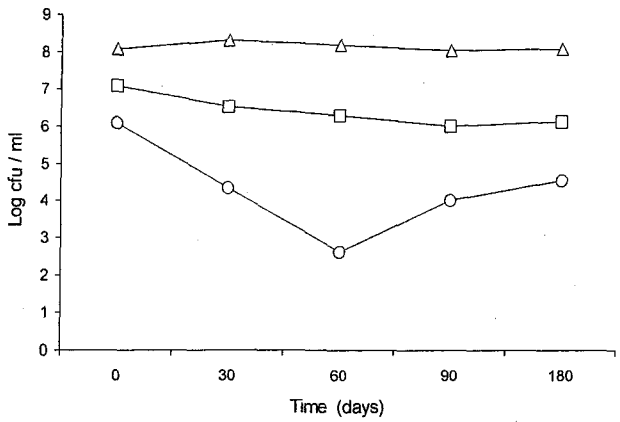

Fig. 2. Starvation-survival response of *E. amylovora* in irrigation water microcosms maintained at 26°C: ○, plate counts on King's B medium; □, viable cell counts and Δ, total cell counts.

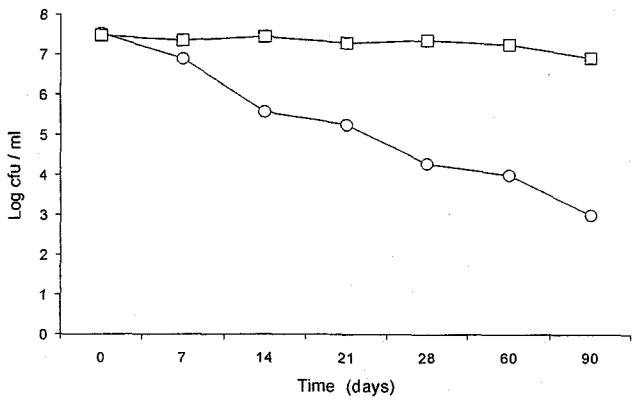

Fig. 3. Temperature effect on the culturability of *E. amylovora* in irrigation water microcosms on King's B solid medium at 26°C (○) and 5°C (□).