Copper Induces a Viable but NonCulturable (VBNC) State in *Erwinia amylovora*

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Keywords: free-copper ions, survival, viability, culturability, resuscitation, pathogenicity, control

Abstract

Copper compounds, used to control *Erwinia amylovora*, have a strong effect on the culturability of plant-associated bacteria. Recent studies have shown that some phytopathogenic bacteria enter into a viable but nonculturable (VBNC) state in the presence of copper. This state, in which cells progressively lose their culturability on non-selective solid media, is considered to be a survival strategy under adverse environmental conditions. To determine whether copper kills or induces a VBNC state in *E. amylovora* cells, mineral medium without copper or supplemented with 0.005, 0.01 and 0.05 mM Cu^{2+} was inoculated with 10^7 cfu/ml of the pathogen. Aliquots were taken regularly for four months, and the total and viable cell counts were determined using the Live/Dead staining kit and culturable cell counts were determined on King's B medium. *E. amylovora* entered into a VBNC state in the presence of the three copper concentrations assayed. It entered faster the VBNC state with increased copper concentration: it entered at days 36, 1 and 0 (immediately after being introduced in the medium) with 0.005, 0.01 and 0.05 mM Cu^{2+} respectively. Afterwards, the restoration of culturability or resuscitation of copper-induced VBNC *E. amylovora* cells was achieved by the addition of different copper complexing agents that could remove the free-copper ions. Dilution studies were also performed to demonstrate that the resuscitated cells came from a true resuscitation and not from the regrowth of any undetectable culturable cell. Finally, the pathogenicity of both VBNC and resuscitated cells was evaluated by inoculation on immature pear fruits. Copper-induced VBNC cells were virulent only for five days while resuscitated cells held their pathogenicity for more than four months. Understanding the effect of copper against *E. amylovora* cells could help to optimize fireblight control strategies.

INTRODUCTION

Copper compounds have been used against fire blight since 1900 (van der Zwet and Keil, 1979) and they are still the most common used strategy for control of bacterial plant diseases, especially since antibiotics are not allowed for most edible crops (Cha and Cooksey, 1991). However, many bacteria have developed resistance mechanisms to the free-copper ions Cu^{2+}, which are the active ingredient in copper compounds (Zevenhuizen et al., 1979; Menkissoglu and Lindow, 1991; Psallidas and Tsiantos, 2000). These mechanisms are sequestration, efflux, synthesis of extracellular binding proteins or copper precipitation (Saxena et al., 2002), but so far, none of these mechanisms have been studied in *E. amylovora*.

Copper, traditionally used as a bactericide in agriculture (van der Zwet and Keil, 1979; van der Zwet and Beer, 1995; Psallidas and Tsiantos, 2000), has been shown to induce a viable but nonculturable (VBNC) state in several plant pathogenic bacteria such as *Agrobacterium tumefaciens* and *Rhizobium meliloti* (Alexander et al., 1999), *Xanthomonas campestris* pv. *campestris* (Ghezzi and Steck, 1999) and *Ralstonia*

Proc. 10th Intl. Workshop on Fire Blight
Eds. C. Bazzi and U. Mazzucchi
Acta Hort 704, ISHS 2006

solanacearum (Grey and Steck, 2001). This state, in which cells progressively lose their culturability on non-selective solid media, is considered to be a survival strategy under adverse environmental conditions (Oliver, 1993). The effectiveness of biocides is often measured by assaying the absence of bacterial growth on a solid medium but the failure of a bacterial cell to produce a colony may not necessarily mean that the cell is dead (Nyström, 2001). Consequently, the ability of plant pathogenic bacteria to enter into a VBNC state by copper should be kept in mind to prevent the underestimation of viable cells, which may still be pathogenic.

Despite the progress in management strategies for fire blight, this disease remains difficult to control (Norelli et al., 2003). In fact, it has been pointed out that *E. amylovora* has the ability to spread and survive in many diverse ways, which could explain the difficulty to get a satisfactory control (Thomson, 2000). To determine whether copper kills or induces a VBNC state in *E. amylovora* cells we have investigated the ability of this bacterium to enter into the VBNC state in the presence of free-copper ions and the pathogenicity of these VBNC cells. Furthermore, we have studied the possible reversion or resuscitation from the nonculturable state of this pathogen and whether the resuscitated cells retain or not their virulence.

MATERIALS AND METHODS

Inoculation of *E. amylovora* in Mineral Medium with Copper

Containers with sterile mineral medium AB (Alexander et al., 1999) supplemented with different copper concentrations (0, 0.005, 0.01 and 0.05 mM Cu^{2+}) were inoculated with 10^7 cfu/ml of two *E. amylovora* strains: CFBP-1430 (French reference strain from the Collection Francaise des Bacteries Phytopathogenes) and IVIA-1892-1 (Spanish strain from the Instituto Valenciano de Investigaciones Agrarias IVIA collection). All these containers were kept in the dark at 26°C throughout the four months of the experiment.

Bacterial Cell Counts

Aliquots of 1 ml were taken regularly from all the containers at various times after inoculation (time zero) and then bacterial counts were determined. Culturable *E. amylovora* cells were counted by plating on King's B solid medium and total and viable cells were determined by fluorescence microscopy using the bacterial viability kit *Bac*Light LIVE/DEAD (Molecular Probes Inc., Eugene, Oreg., USA).

Restoration of Culturability or Resuscitation of Copper-induced VBNC Cells

Confirmation of the VBNC state hypothesis requires the recovery of culturable cells from a population of nonculturable cells (Bogosian and Bourneuf, 2001). Aliquots of 1 ml from all the containers were taken at different times through the experiment. Chelating compounds such as EDTA and citric acid, the amino acid asparagine and King's B liquid medium were added to the aliquots to remove the free-copper ions from AB medium. EDTA, citric acid and asparagine were added in stochiometric amount with copper concentration while King's B was diluted 1/10. After 48 h of shaking incubation at 26°C, the culturability was determined on King's B solid medium.

Dilution Studies: Demonstration of Resuscitation

To evaluate if the reappearance of culturable cells after the removal of free-copper ions resulted from true resuscitation of the copper-induced VBNC cells or from regrowth of a few culturable but undetected cells, dilution studies were performed according to Whitesides and Oliver (1997). Briefly, aliquots of 1 ml from the containers with 120 days old copper-induced VBNC cells in AB medium with 0.01 mM Cu^{2+} were serially ten-fold diluted to reduce the probability (p) of any initial culturable cell down to less than 0.0000001 cfu/ml. Then, King's B broth was added to each dilution and after 48 h of shaking incubation at 26°C, the culturability was examined on King's B plates.

Pathogenicity Assays of Copper-induced VBNC and Resuscitated *E. amylovora* Cells

Copper-induced VBNC and resuscitated cells of the bacterium were inoculated on immature pear fruits as described by López (2004) at different time intervals during four months. King's B grown *E. amylovora* cells and AB medium with copper were used in all the inoculation assays as a positive and a negative control, respectively. After incubation at 26°C the production of symptoms was examined daily for two weeks.

RESULTS

Copper Induces a VBNC State in *E. amylovora*

The total, viable and culturable cell counts performed throughout four months clearly indicated that *E. amylovora* enters into a VBNC state induced by copper (Fig. 1). Total and viable cell counts remained relatively constant at the initial levels (10^8-10^9 cells/ml) in all the cases, independently of the copper concentration (Fig. 1). However, the culturability of the bacterium decreased in different ways depending on the copper concentration. In copper-free AB medium, the culturability decreased slightly but remained at 10^4 cfu/ml until the end of the experimental period (Fig. 1A). In the presence of copper ions, the culturability of *E. amylovora* went down quickly below the detection level (<1 cfu/ml) and cells became nonculturable in spite of the high numbers of viable cells. This VBNC fraction of the bacterial population can be determined by subtracting culturable from viable cell counts (Roszak and Colwell, 1987). Thus, most of the bacterial population (87.5-94.4%) enter into VBNC state in the presence of the three copper concentrations assayed. The time of entry into the VBNC state was much earlier as the concentration of copper was higher (days 36, 1 and 0 for 0.005, 0.01 and 0.05 mM Cu^{2+}, respectively) (Fig. 1).

Resuscitation of Copper-induced VBNC *E. amylovora* Cells

Copper complexing agents were added to remove the Cu^{2+} ions present in the AB medium. In all cases, their addition was effective to restore the culturability of copper-induced VBNC cells, but the ability to recover such cells varied depending on the amount of time *E. amylovora* had spent into the VBNC state (Fig. 2). EDTA and citric acid were effective for the first 18 days while asparagine was effective up to 80 days. The highest numbers of resuscitated cells were reached with King's B liquid medium, even when these resuscitated cells were recovered more than four months later (Fig. 2).

To demonstrate that the restoration of culturability achieved was a true resuscitation and not a regrowth, dilution studies were performed before the addition of the King's B liquid medium. Resuscitated cells were recovered from all the dilutions even when the probability to find a culturable cell was as low as 0.0000001 cfu/ml.

Pathogenicity of Copper-induced VBNC and Resuscitated *E. amylovora* Cells

While *E. amylovora* cells in free-copper AB medium were always virulent throughout the experimental period, copper-induced VBNC cells held their pathogenicity for the first five days. The recovery of the ability to cause symptoms was only reached when culturability was regained. Thus, the pathogenicity of resuscitated cells was only observed during 18 days with EDTA or citric acid; 80 days for asparagine, and more than four months in the case of King's B liquid medium.

DISCUSSION

In this work, it has been shown, for the first time, that copper induces a VBNC state in *E. amylovora*, like it has been described for other plant pathogenic bacteria (Alexander et al., 1999; Ghezzi and Steck, 1999; Grey and Steck, 2001). In general, *E. amylovora* cells entered into the VBNC state induced by copper ions earlier and in a higher proportion than the other plant pathogens studied. Furthermore, in contrast with these studies, the fire blight bacterium was able to regain its cellular activity after a longer time in the VBNC state. According to this, the VBNC state could be a part of the life

cycle of *E. amylovora* under adverse environmental conditions unknown to date. Thus, this physiological cell state could be involved in the recurrent infections of fire blight, and therefore, be responsible of its difficult control. In fact, the occurrence of phytopathogenic bacterial cells in the VBNC state could have serious implications in plant pathology, since epidemiological studies are usually based on plate counts of culturable cells (Wilson and Lindow, 2000).

Since the resuscitation of nonculturable cells is considered the keystone of the VBNC state hypothesis (Bogosian and Bourneuf, 2001), demonstration of real resuscitation is absolutely needed. Kell et al. (1998) proposed that in order to differentiate resuscitation from regrowth, it is imperative to determine the probability that a given sample contains any culturable unit prior to resuscitation. These authors also pointed out that to demonstrate that the contribution of regrowth has been excluded, it is necessary to decide on the statistical limits that are acceptable; for example, $p<0.01$ that a single viable cell was present. Such conditions are fulfilled in this work. Resuscitated cells were recovered even when $p<0.0000001$, indicating that it must be due to true resuscitation and not to regrowth of undetectable culturable cells (Whitesides and Oliver, 1997). However, regardless of whether restoration of culturability was a consequence of true resuscitation or regrowth, the most practical important fact is that in this work the undetectable cells of the pathogen retained their virulence, as it was stated by Oliver (1993). Thus, the maintenance of pathogenicity of the resuscitated *E. amylovora* cells during more than four months could be one reason of the reappearance of fire blight outbreaks after copper treatments.

The copper concentrations used in this work were chosen based on a previous report (Zevenhuizen et al., 1979) that stated that 0.005 mM Cu^{2+} was the minimum concentration required to inhibit bacterial growth in minimal medium. Despite the high copper concentrations used in agriculture, by comparison to the low concentrations used in this study, the present results should be taken into account since copper ions are significantly complexed in natural environments. This could favour the survival of *E. amylovora* in soils or on leaves, where it is feasible that copper ions would be complexed after application of copper compounds.

Overall, the present results demonstrate the induction of the VBNC state in *E. amylovora* by copper and the ability of *E. amylovora* to recover culturability and pathogenicity in the presence of copper complexing agents. Further studies on the interaction between copper and *E. amylovora* and the role of VBNC state in its survival and life cycle are needed. This knowledge will improve the understanding of the epidemiology and the optimization of control strategies of fire blight.

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Figures

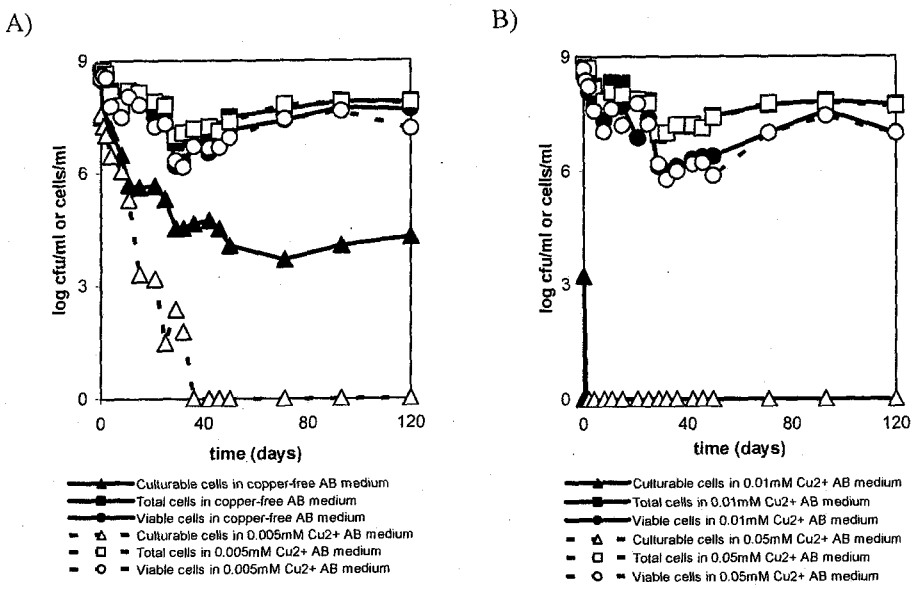

Fig. 1. Total, viable and culturable cell counts of *E. amylovora*. A) Copper-free AB medium vs. AB medium supplemented with 0.005 mM Cu^{2+}. B) AB medium supplemented with 0.01 mM Cu^{2+} vs. AB medium supplemented with 0.05 mM Cu^{2+}.

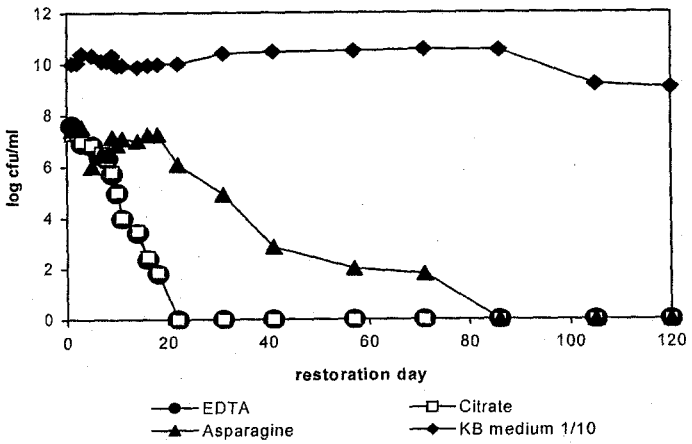

Fig. 2. Resuscitation of copper-induced VBNC *E. amylovora* cells by the addition of: chelating compounds (EDTA and citric acid), the amino acid asparagine and King's B liquid medium. EDTA, citric acid and asparagine were added in stochiometric amount with copper concentration while King's B was diluted 1/10.