Journal of Invertebrate Pathology 113 (2013) 115–121

Contents lists available at SciVerse ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Mode of action of *Pseudomonas fluorescens* strain CL145A, a lethal control agent of dreissenid mussels (Bivalvia: Dreissenidae)

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ARTICLE INFO

Article history:
Available online 9 January 2013

Keywords:
Zebra mussel
Dreissena polymorpha
Biological control
Biopesticide
Pseudomonas fluorescens
Hemocyte infiltration

ABSTRACT

Pseudomonas fluorescens strain CL145A (*Pf*-CL145A) has demonstrated promise as an efficacious and selective agent for the control of macrofouling *Dreissena* spp. mussels. Herein, we report trials to investigate the mode of action of this biocontrol agent against *Dreissena polymorpha*, the zebra mussel. Exposure to dead *Pf*-CL145A cells achieved the same temporal pattern and percentage mussel mortality as did live cells, thereby excluding infection as the possible lethal mode of action. Histological analysis revealed pathologies consistent with the cause of death being intoxicating natural products associated with *Pf*-CL145A cells. Irrespective of whether the mussels were exposed to live or dead *Pf*-CL145A cells, examination of tissues from histological sections revealed that: (1) at the end of the 24-h treatment period there was massive hemocyte infiltration into the lumina of both the digestive gland and stomach; and (2) mussel deaths occurred following lysis and necrosis of the digestive gland and sloughing of stomach epithelium. These trials provide strong evidence that the lethal mode of action of *Pf*-CL145A is intoxication.

1. Introduction

Microorganisms produce a vast array of natural products which can be exploited for human benefit, including antibiotics, unique enzymes for industrial applications, and biopesticides. The use of microbial metabolic products has a clear record of commercial success and environmental safety in the control of invertebrate pests in North America, as well as globally (Rodgers, 1993). In this regard, trials evaluating the bacterium *Pseudomonas fluorescens* strain CL145A (*Pf*-CL145A) have demonstrated its significant potential as a novel, highly selective, and efficacious agent for the control of zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*) (Molloy et al., in press, 2013). These dreissenid mussels are invasive species that have caused significant ecological and economic impacts in North America since their introduction from Europe in the 1980s (Mackie and Claudi, 2009). Herein, we present evidence that the mode of action of strain *Pf*-CL145A is intoxication, not infection, and that mussel deaths occur following lysis and necrosis of the digestive gland and sloughing of stomach epithelium.

2. Materials and methods

2.1. Trials to determine the relative lethality of live versus dead bacterial cells

Duplicate laboratory trials (hereafter referred to as Trials #1 and #2) were conducted to examine the relative efficacy of live versus dead *Pf*-CL145A cells in causing zebra mussel mortality. The rationale of this experimental approach was that if exposure to dead cells achieved the same mussel mortality as live cells, this would be clear evidence that mussel death was not due to infection by *Pf*-CL145A.

To produce the bacteria required for these trials, 10-ml tubes containing 6 ml of tryptic soy broth (TSB) (Difco) were each inoculated with 0.1 ml stock frozen *Pf*-CL145A culture and incubated at 26 °C under static conditions for 42 h. Tubes were subsequently used to inoculate 250-ml flasks (1 tube per flask) each containing 100-ml TSB and incubated at 26 °C under static conditions for 72 h (i.e., until early stationary phase). Final whole cultures were centrifuged (30 min at 827*g*) in 50-ml batches, supernatants were discarded, and cell fractions (CF) were resuspended in dilution water (80 ppm KH_2PO_4, 405.5 ppm $MgCl_2 \cdot 6H_2O$ in deionized water, pH-adjusted to 7.2 with NaOH). The optical density of the CF inoculum was determined by taking an absorbance reading (spectrophotometer, $\lambda = 660$ nm) from each of 3 separate samples of the

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http://dx.doi.org/10.1016/j.jip.2012.12.013

CF. Based on an absorbance equation developed during previous trials, the optical density of the CF was then used to calculate the volume of CF inoculum required to treat the mussels. To produce the dead-cell inoculum needed for this trial, a portion of the CF inoculum was exposed to 57 krad of gamma radiation with cesium 137 chloride over a 3-h period. This resulted in ⩾99.9994% cell kill as calculated from the ratio of live cells in unexposed controls to exposed treatments using standard cfu/ml plate counts in Petri dishes of tryptic soy agar (Difco).

Zebra mussels were collected from the Mohawk River (Crescent, New York) and were stored at 5–6 °C in aquaria containing aerated, filtered, unchlorinated tap water. Mussels (6–11 mm in length) were acclimated to trial temperatures for a week prior to treatment. Both trials were conducted in 500 ml of aerated, unchlorinated tap water in 1-L glass jars with 3 replicates of 25 mussels plus an untreated control. Mussels were exposed for 72 h to 100 ppm (mg cell mass per L) of either live or dead cells. Based on our standard culturing yields, the 500 ml of water in the 3 jars being treated at 100 ppm with live cells contained 6×10^7 live cells per ml.

Following treatment, mussels were transferred from the jars to glass Petri dishes containing oxygenated tap water, and then were held for 11 days posttreatment. Dead mussels were removed and water was changed daily. All testing was at 20(±1) °C.

2.2. Trials using histological analysis to determine mode of action

To further investigate the mode of action, the histological appearance of mussel tissues and organs following treatment with *Pf*-CL145A cells was examined. Separate trials were conducted with live and dead *Pf*-CL145A cells.

2.2.1. Histological analysis of mussels treated with live bacterial cells

The bacteria used in this live-cell trial were produced as outlined in Section 2.1. The zebra mussels (mean length ± SD [n = 20] = 7.7 ± 1.2 mm) were collected from the Mohawk River (Crescent, New York) and held in aerated, filtered, unchlorinated tap water at 7(±1) °C for 28 days. Mussels were then transferred to an aquarium containing aerated, filtered, unchlorinated 7 °C tap water that was allowed to gradually warm to 23 °C over a 3-day period. Thirty mussels were then placed in each of 3 1-L glass jars containing 250 ml of hard, synthetic, freshwater (hereafter referred to as "hard water") (192 ppm $NaHCO_3$, 120 ppm $CaSO_4{\cdot}2H_2O$, 120 ppm $MgSO_4$, 8 ppm KCl; United States Environmental Protection Agency, 2002). The next day the water in each jar was replaced with a total of 500 ml of hard water, and aeration was initiated. Two of the jars were then treated at 300 ppm (these mussels hereafter are referred to as *Treated Mussel Groups #1 and #2*), and the third jar served as the untreated control (hereafter referred to as the *Untreated Mussel Group*). After the 24-h bacterial treatment, the mussels in each jar were transferred to separate Petri dishes containing oxygenated hard water. All testing was at 23(±1)°C.

Treated Mussel Group #1: This group of mussels provided specimens solely for assessment of the condition of mussel tissues and organs *prior to death*. Twenty-four live individuals were randomly selected from this group and processed for histological examination, i.e., 8 mussels immediately removed after their initial placement in the Petri dish and another 8 mussels 1 and 2 days later.

Treated Mussel Group #2: This group of mussels provided specimens solely for *postmortem* histological analysis. Water was changed and dead mussels were removed daily from this Petri dish. The first 8 of these dead mussels were processed for histological examination.

Untreated Mussel Group: To obtain observations of the normal histological appearance of the tissues and organs of mussels that had never been exposed to the bacteria, 15 of the 30 untreated control mussels were randomly removed from their Petri dish for fixation, i.e., 5 mussels immediately removed after their placement in the Petri dish and another 5 mussels 1 and 2 days later. During the entire 9-day posttreatment period, hard water in this control dish of untreated control mussels was changed daily, and any dead mussels removed.

2.2.2. Histological analysis of mussels treated with dead bacterial cells

The bacteria used in this dead-cell trial were cultured in fermentation units at the Center for Biocatalysis and Bioprocessing at the University of Iowa (Iowa City, Iowa) and harvested during early stationary phase. Final whole cultures were centrifuged, supernatants were discarded, and CF was stored at −80 °C. To produce the dead bacteria for this trial, the frozen CF was subsequently irradiated by electron beam (0.95 Mrad) at E-Beam Services, Inc. (Cranbury, New Jersey). When portions of the frozen CF were thawed, it was determined that the radiation had resulted in a >99.9999999% reduction in cell viability as calculated from the ratio of live cells in unexposed controls to exposed treatments using standard cfu/ml plate counts in Petri dishes of tryptic soy agar (Difco). The optical density of the CF inoculum was determined by taking an absorbance reading (spectrophotometer, λ = 660 nm) from each of 3 separate thawed samples of the frozen CF. Based on the absorbance equation developed, the optical density of the CF was then used to calculate the volume of CF inoculum required to treat the mussels. A portion of the frozen CF was then thawed and suspended in dilution water immediately prior to use in this trial.

Zebra mussels from Hedges Lake (Cambridge, New York) were collected and held in an aquarium containing aerated, filtered, 21(±1) °C water (mix of 50% unchlorinated tap water and 50% Hedges Lake water) for 11 days prior to the test. Mean (±SD, n = 20) length of mussels used in the test was 14.5(±4.3) mm. Twenty-seven mussels were placed in each of 6 1-L glass jars containing 250 ml of hard water. The next day the hard water in each jar was replaced with a total of 500 ml, and aeration was initiated. Three of the jars were then treated at 200 ppm, and 3 jars served as untreated controls. To verify ingestion of the bacterial cells and assess host tissue condition early in the treatment period, 2 mussels from each jar were removed after 2 h and processed for histological analysis. After 24 h of treatment, the remaining 25 mussels in all jars were transferred to fresh jars containing 500 ml of oxygenated hard water and aeration continued. Dead and moribund mussels were removed and hard water was changed daily during the 13-day posttreatment period. The first 8 of these mussels that were removed from each replicate jar were fixed for histological processing. All testing was at 21(±1) °C.

2.2.3. Histological protocol

All mussels undergoing histological analysis in this study were fixed in 10% neutral buffered (sodium phosphate) formalin, dehydrated in a graded series of alcohols and toluene, and embedded in paraffin. Serial sections (5 μm thick) were cut, stained with hematoxylin and eosin, and examined by light microscopy (≤1000×).

3. Results

3.1. Efficacy of live versus dead bacterial cells

There was no statistical difference in mussel mortality whether mussels were treated with live or dead *Pf*-CL145A cells ($p < 0.05$, analysis by t-test of angular transformed percent mortality data). Mean mortality (±SD, n = 3) in Trials #1 and #2 with live versus