INTERNATL MICROBIOL (1999) 2:111–114

RESEARCH ARTICLE

Nancy I. López[1]
Ana S. Haedo[2]
Beatriz S. Méndez[2]

[1]Department of Biological Sciences, Faculty of Sciences, University of Buenos Aires, Argentina
[2]Department of Biological Chemistry, Faculty of Sciences, University of Buenos Aires, Argentina

Evaluation of *Xanthomonas campestris* survival in a soil microcosm system

Received 19 November 1998
Accepted 15 March 1999

Correspondence to:
Beatriz S. Méndez. Departamento de Química Biológica. Facultad de Ciencias Exactas y Naturales. Ciudad Universitaria. Pabellón 2. 1428 Buenos Aires. Argentina.
Tel.: +54-11-45763334. Fax: +54-11-45763342.
E-mail: bea@qb.fcen.uba.ar

Summary *Xanthomonas campestris* pv. *campestris* is a pathogen of cruciferous plants. We studied the survival of the wild type strain and mutant derivatives which are deficient in exopolysaccharide (EPS) or in extracellular protease synthesis in soil microcosms in order to test the hypothesis that, in this environment, adherence to soil particles and scavenging of nutrients are very important strategies for bacterial survival. In sterile soil microcosms, differences in survival were only observed between the EPS producer and its mutant. In non-sterile soil experiments, survival of Prt^- mutant was similar to EPS^- mutant, suggesting that both characteristics have a strong influence in survival in the presence of the natural bacterial community. Bacterial decrease represented by the slope of regression lines was higher in non-sterile soil microcosms due to the influence of biotic interactions.

Key words *Xanthomonas campestris* · Exopolysaccharides · Extracellular proteases · Bacterial survival · Soil microcosms

Introduction

Xanthomonas campestris pv. *campestris* is a pathogen on cruciferous plants, causing the disease called black rot [13]. The infection can occur from infested soil. However, only a few works have intended to clarify the influence of the soil on the ecology of *X. campestris*. Schaad and White [13] have shown that *X. campestris* survived in soil for a relatively short period of time when unprotected by host tissues, and depending upon the season of the year. The pathogen could be recovered from soil infestations during a period of 42 days in winter, but only during 14 days in summer. Habte and Alexander [7] proposed protozoa as the agents responsible for the decline of *X. campestris* in soil. In Argentina [6], it was shown that the survival of this species was related to soil moisture and temperature. By using a strain of *Xanthomonas campestris* pv. *campestris* genetically engineered to bioluminesce, Shaw et al. [14] demonstrated that the bacteria could be detected in plant samples and in the rhizosphere up to 6 weeks after inoculation.

Due to the fact that soil is an heterogeneous, discontinuous and structured environment, soil bacteria could face different stresses caused by limitations in nutrient availability or by physical or chemical factors which fluctuate in time [18]. The ability of bacteria to maintain high cell numbers in different environments involves several strategies which depend on the characteristics of each species [8].

Bacterial capability for exopolysaccharide (EPS) production has been involved in several mechanisms which include the selective and non-selective adhesion to inert surfaces, animal tissues and plant cell walls. EPS also protect bacterial cells from dehydration and modify the soil by promoting soil particle adhesion thus reducing soil erosion [11]. For these reasons, bacterial capability for EPS production could represent an advantage for survival both as free soil bacteria and in association with the host.

Xanthomonas campestris pv. *campestris* produces a range of extracellular enzymes, including protease, polygalacturonate lyase, endoglucanase and amylase, that are necessary to establish a host-pathogen interaction [16]. These enzymes are also secreted by microorganisms to scavenge for nutrients.

The understanding of factors affecting the survival of pathogens and their mutant derivatives in soil is relevant for the design of biocontrol strategies. In this article we analyze the importance of EPS and extracellular protease production in the survival of *Xanthomonas campestris* pv. *campestris* as free bacteria in soil by using mutants and soil microcosms. Microcosms experiments are adequate for this task because they are simpler than field experiments and they avoid unmanageable complexity.

Materials and methods

Soil samples Soil samples were collected from an experimental field at the Faculty of Agronomy (University of Buenos Aires). Neither pesticides nor herbicides were applied to this soil and only natural organic fertilizers were used. Samples were taken from the top 5 cm of the A horizon. Water and organic matter contents were measured as previously described [9]. pH was determined by mixing soil with 0.01 M $CaCl_2$ (1:1, v/v) [8].

Bacterial strains *Xanthomonas campestris* pv. *campestris* strains 8004, 8004::Tn*5*$_{70}$, 8004::Tn*5*C were used in this study. Strain 8004 is a pathogen, rifampicin resistant [4, 17]. Strain 8004::Tn*5*$_{70}$ is a variant EPS$^-$, nonpathogenic derived by insertion of Tn*5* [2]. Tn*5* was located 15 bp upstream of the initiation site of *gumB* gene (Vojnov AA, Ph.D. Thesis, University of Buenos Aires, 1996). Strain 8004::Tn*5*C is a protease-synthesis deficient mutant (Prt$^-$) derived from 8004 strain by insertion of Tn*5* [15, 16].

Microcosm design Microcosms were prepared according to Lafuente et al. [8], and modified as follows: samples of 15 g of soil were placed in 10 cm Petri dishes and distributed in a layer. Sterile distilled water was added to the soil to reach a final moisture content of 40% (v/wt). Two series of ten microcosms were prepared, one contained the wild type strain and the other one contained the mutant strain for each phenotype analyzed. Experiments were performed using sterile soil and non-sterile soil. Each experiment was repeated twice. Microcosms containing non-sterile soil were preincubated at 30°C, prior to the experiment, to allow the indigenous microorganisms to reach an equilibrium state, avoiding a thermal artifact at time zero of the experiment [10]. In the sterile microcosms, soil was dried at 50°C for 3 days, sieved through a 0.4 cm sieve and sterilized in small paper packets by autoclaving three times on consecutive days at 121°C for 60 min [8]. Bacteria were grown with shaking at 30°C in Luria-Bertani (LB) broth plus 25 µg/ml of rifampicin (Rif) or Rif plus 25 µg/ml of kanamycin (Km) to the late exponential phase, yielding a final cell concentration of 10^7 colony-forming units (cfu) per ml. Cells from 100 ml cultures were harvested by centrifugation and resuspended in 5 ml of sterile saline solution (0.9% NaCl), 0.5 ml of the resulting bacterial suspensions were dropped onto the center of the Petri dish, and the soil was not disturbed. Microcosms plates were sealed with Parafilm®, and incubated at 30°C.

Bacterial counts Bacteria were counted immediately after the inoculum was added (day 0) and then once or twice a week during about 20 days. In each counting event, one microcosm containing the wild-type strain and other containing the mutants were analyzed. For cell extraction, the soil of each Petri dish was suspended in 85 ml of 0.1% of sterile tetrasodium pyrophosphate solution (0.1%) and the suspension was subjected to magnetic stirring for 15 min. Suspensions from sterile soil microcosms were counted on LB-agar plates while LB-agar plates plus 25 µg/ml of Rif and 25 µg/ml of Km, supplemented with 100 mg of cycloheximide per liter were used for bacterial suspensions from non-sterile soil microcosms. The plates were incubated at 30°C during 48 h. Bacteria were enumerated by viable triplicate plate counts.

Statistics Linear regression analysis was used to estimate the rate of decrease in log number of cfu per g of dry soil over time. The aim of this analysis was not to make strong predictions but rather to compare survival of the strain 8004 and its EPS$^-$ and Prt$^-$ derivatives under different soil conditions. The statistical significance of the difference between the slopes of the regression lines was tested using analysis of covariance. These analyses were performed using Program 1V of the BMDP statistical software (1993).

Results

Survival of *Xanthomonas campestris* pv. *campestris* 8004 and its EPS$^-$ and Prt$^-$ derivatives were compared by two different approaches. In one case, sterile soil was used to evaluate the influence of abiotic parameters, whereas, in the second case, the experiments were performed with soil containing indigenous microorganisms to determine the influence of biological interactions. For this purpose, we used strains marked with antibiotic resistance to avoid the problem of plating the local bacterial community. No resistant bacteria or fungi were detected in soil samples at the rifampicin, kanamycin and cycloheximide concentrations used. The mean of soil water and organic matter content were 18% and 8% respectively and pH was 7.3. According to viable plate counts soil samples contained 5.8×10^8 cfu per g of dry soil.

Survival of EPS producing strain in sterile soil In sterile soil experiments, differences between strains 8004 and 8004::Tn*5*$_{70}$ were observed. Survival of the wild-type strain was higher than that observed for EPS$^-$ mutant. Bacterial numbers decreased during the experiment. The EPS$^-$ strain showed a strong decrease in the number of viable cells (Fig. 1). The experiment was repeated twice showing the same survival characteristics under these conditions. According to analysis of covariance, the difference in slopes of the regression lines were highly significant in both experiments ($P < 0.001$), and survival was always lower in the EPS$^-$ strain as compared with the wild type strain. This analysis allowed us to estimate that the decrease in cell number of the EPS$^-$ mutant was 2 orders of magnitude or higher when compared with the wild type's at the end of the experiments.

Comparison between wild type and protease defective strains in sterile soil No differences in survival capability between the wild-type strain and the Prt$^-$ strains were observed, cell numbers decreased until 2×10^5 cfu per g of dry soil and

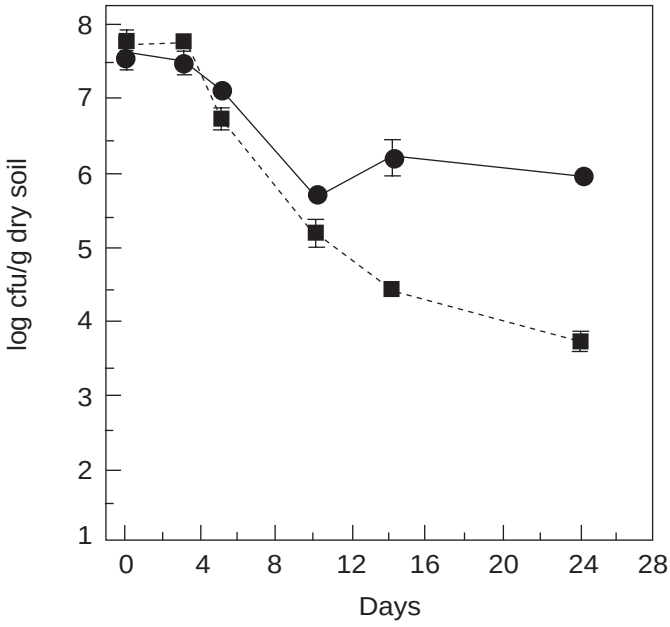

Fig. 1. Survival of *Xanthomonas campestris* pv. *campestris* in sterile soil microcosms. Values represent mean ± 1 SD of three replicates log transformed data. Symbols: ● 8004 strain, j 8004::Tn*5*$_{70}$ strain

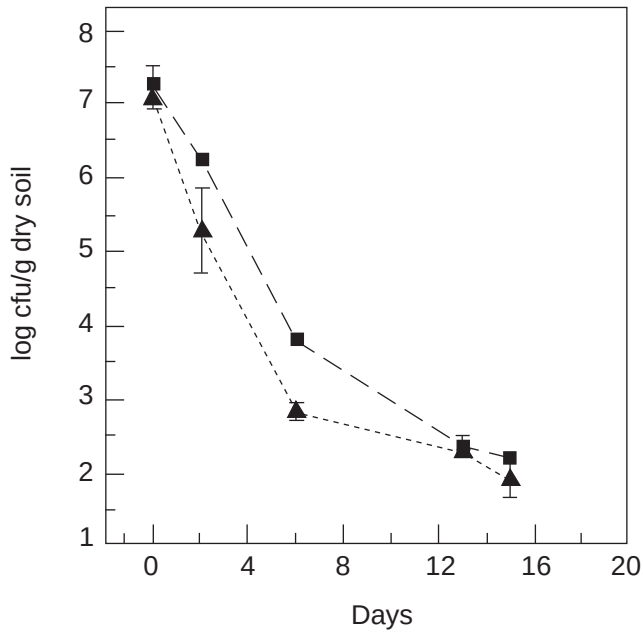

Fig. 3. Survival of *Xanthomonas campestris* pv. *campestris* in non-sterile soil. Values represent mean ± 1 SD of three replicates log transformed data. Symbols: j 8004::Tn*5*$_{70}$ strain, ˙ 8004::Tn*5*C strain

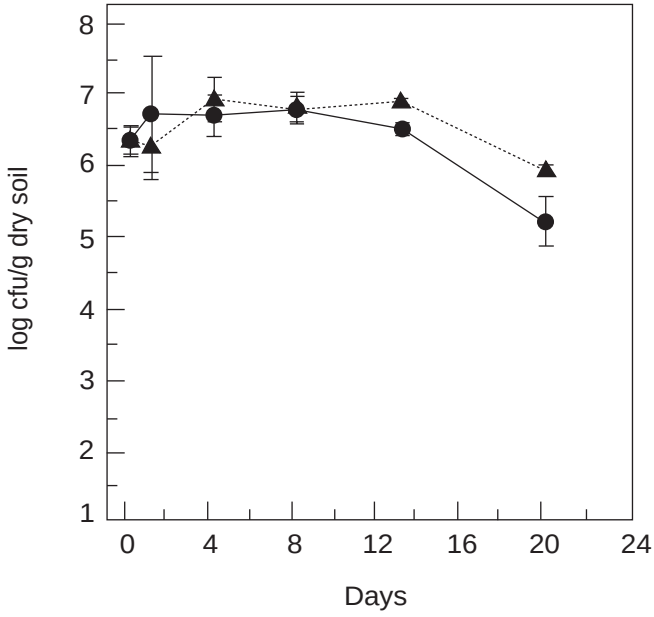

Fig. 2. Survival of *Xanthomonas campestris* pv. *campestris* in sterile soil. Values represent mean ± 1 SD of three replicates log transformed data. Symbols: ● 8004 strain, ˙ 8004::Tn*5*C strain

8 x 10^5 cfu per g of dry soil respectively after 20 days (Fig. 2). Similar results were obtained in the previous experiment for the wild type strain (Fig. 1). Linear regression was not adequate for the description of the wild type strain and the protease deficient strain survival pattern because the slopes of the curves were not significantly different from zero (P >0.05). This analysis allowed us to suggest that deficiency in protease activity was not a disadvantage for survival in sterile soil microcosms. Based on these results we used both the EPS$^-$ and the Prt$^-$ mutants to compare the influence of these characteristics on the survival in non-sterile soil microcosms.

Survival of EPS$^-$ strain and Prt$^-$ strains in non-sterile soil microcosms Bacterial numbers decreased during the whole experiment for both mutant strains (Fig. 3). This experiment was repeated twice showing the same survival pattern. Thus in non-sterile soil the deficiency in the synthesis of extracellular protease or EPS affected bacterial survival, suggesting that biological interactions may have a strong influence in survival, and that protease deficiency constitutes a disadvantage under such conditions, similar to EPS deficiency. The analysis of covariance showed a similar and non-significant (P >0.05) decrease pattern for both strains but, in this case, the Prt$^-$ strain showed a stronger decrease than in the sterile microcosm experiment.

Comparison of survival patterns between raw and sterile soil at day 15 of the experiments, showed a 100-fold reduction for the EPS$^-$ mutant (Figs. 1 and 3). By contrast, very high reduction (10^5-fold) in the viable cell counts was observed for the Prt$^-$ mutant when comparing both microcosms (Figs. 2 and 3).

Discussion

The ecology of plant pathogenic bacteria has not been clearly established because of the lack of a sensitive system to trace the bacteria in this environment [13]. Methods based on

bacterial isolation from soil using semi-selective media [3, 5] are not useful for debris-infected soils due to the overgrowth of non targeted naturally occurring bacteria. Therefore, new approaches based on genetic techniques have been implemented.

Bioengineered microorganisms containing a luciferase gene [3] were used by Arias et al. [1] for monitoring a bioluminescent strain of *Xanthomonas campestris* pv. *campestris* in debris inoculated soil.

The approach used in this work, microcosm systems inoculated with wild type and mutant strains, can be used to predict how bacteria behave in natural habitats. Our results showed that the differences observed between both soil microcosms (sterile and non-sterile) were due to biological interactions and changes in soil conditions. Biological interactions were represented by competition with other bacteria or higher organisms, and by predation. Visual inspection of non-sterile soil microcosms showed seed germination after 2–3 days of incubation. Plants in the numbers of 1 to 3 per microcosm died approximately 4 days after the experiments started. This observation constitutes an evidence of the simultaneous demand of resources by bacteria and higher organisms in non-sterile conditions. The construction of sterile soil microcosms implies changes in the chemical and spatial properties of soil. With the design employed it is impossible to determine the relative importance of biotic and abiotic interactions in bacterial survival, but we suggest that the absence of competition is the primary advantage of bacteria for increasing their survival. Survival experiments on *X. campestris* pv. *campestris* showed that the ability to produce exopolysaccharides is advantageous in soil environments without biological interactions. Bacteria deficient in protease activity survive in the same way as the wild type strain without competition. However, in the presence of the natural microbial communities this consideration is altered.

This kind of approach was also used by O'Garro et al. [12] to evaluate the role of the *avrBs1* avirulence gene in the survival of *X. campestris* pv. *vesicatoria* in soil and detached leaf tissue.

Therefore, the validation of bacterial plant pathogens survival in a variety of conditions and by a combination of different techniques is necessary for predictive purposes in the design of biocontrol strategies.

Acknowledgments We thank Ing. Agr. Silvia Moccia and Chair of Horticulture (Faculty of Agronomy, University of Buenos Aires) for kindly allowing us to take samples for this study. We are grateful to Jimena Ruiz and Mónica Giacomodonato for technical assistance. This work was supported by grants of the University of Buenos Aires. B.S.M. and N.I.L. are career investigators from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

References

1. Arias RS, Mochizuki G, Fukui R, Alvarez AM (1996) Most probable number method to enumerate bioluminescent *Xanthomonas campestris* pv. *campestris* in soil. Soil Biol Biochem 28:1725–1728
2. Barrère GC, Barber CE, Daniels MJ (1986) Molecular cloning of genes involved in the production of the extracellular polysaccharide xanthan by *Xanthomonas campestris* pv. *campestris*. Int J Biol Macromol 8:372–374
3. Cebolla A, Ruiz-Berraquero F, Palomares AJ (1993) Stable tagging of *Rhizobium meliloti* with the firefly luciferase gene for environmental monitoring. Appl Environ Microbiol 59:2511–2519
4. Daniels MJ, Barber CE, Turner PC, Cleary WG, Sawczyc MK (1984) Isolation of mutants of *Xanthomonas campestris* showing altered pathogenicity. J Gen Microbiol 130:2447–2455
5. Fukui R, Arias RS, Alvarez R (1994) Efficacy of four semi-selective media for recovery of *Xanthomonas campestris* pv. *campestris* from tropical soils. J Appl Bacteriol 77:534–540
6. Graham JH, Gottwald TR, Civerolo EL, McGuire RG (1989) Population dynamics and survival of *Xanthomonas campestris* in soil in citrus nurseries in Maryland and Argentina. Plant Dis 73:423–427
7. Habte M, Alexander M (1975) Protozoa as agents responsible for the decline of *Xanthomonas campestris* in soil. Appl Microbiol 29:159–164
8. Lafuente R, Maymó-Gatell X, Mas-Castellà J, Guerrero R (1996) Influence of environmental factors on plasmid transfer in soil microcosms. Curr Microbiol 32:213–220
9. López NI, Duarte CM, Vallespinós F, Romero J, Alcoverro T (1995) Bacterial activities in seagrass (*Posidonia oceanica*) sediments. J Exp Mar Biol Ecol 187:39–49
10. López NI, Floccari ME, Steinbüchel A, García AF, Méndez BS (1995) Effect of poly(3-hydroxybutyrate) (PHB) content on the starvation-survival of bacteria in natural waters. FEMS Microbiol Ecol 16:95–102
11. Morris VJ, Miles MJ (1986) Effect of natural modifications on the functional properties of extracellular bacterial polysaccharides. Int J Biol Macromol 8:342–348
12. O'Garro LW, Gibbs H, Newton A (1997) Mutation in the *avrBs1* avirulence gene of *Xanthomonas campestris* pv. *vesicatoria* influences survival of the bacterium in soil and detached leaf tissue. Phytopathology 87:960–966
13. Schaad NW, White WC (1974) Survival of *Xanthomonas campestris* in soil. Phytopathology 64:1518–1520
14. Shaw JJ, Dane F, Geiger D, Kloepper JW (1992) Use of bioluminescence for detection of genetically engineered microorganisms released into the environment. Appl Environ Microbiol 58:267–273
15. Tang JL, Gough CL, Barber CE, Dow JM, Daniels MJ (1987) Molecular cloning of protease gene(s) from *Xanthomonas campestris* pv. *campestris:* expression in *Escherichia coli* and role in pathogenicity. Mol Gen Genet 210:443–448
16. Tang JL, Liu YN, Barber CE, Dow JM, Wootton JC, Daniels MJ (1991) Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar *campestris.* Mol Gen Genet 226:409–417
17. Turner P, Baber C, Daniels M (1984) Behaviour of the transposons Tn*5* and Tn*7* in *Xanthomonas campestris* pv. *campestris*. Mol Gen Genet 195:101–107
18. van Elsas JD, van Overbeek LS (1993) Bacterial responses to soil stimuli. In: Kjelleberg S (ed) Starvation in Bacteria. New York: Plenum Press, pp 55–79