IN VITRO GROWTH AND STORAGE OF TWO SUBSPECIES OF *Clavibacter xyli*[1]

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Resumen

Fueron determinadas curvas de crecimiento en medio líquido y longevidad in vitro *a −20°C y −80 C para las cepas F-1 de* Clavibacter xyli *subsp.* xyli *(raquitismo de la caña de azúcar) y FB-1 de* C. xyli *subsp.* cynodontis *(raquitismo de Bermuda grass), ambas de Florida. El tiempo de generación para F-1 y FB-1 fue estimado a 48 h y 24 h, respectivamente. La población de células viables de F-1 disminuyó de 3.5 x 10^7 a 66 colonias/ml y a 7.4 x 10^4 colonias/ml después de haber sido mantenida durante 120 días a −20°C y −80°C, respectivamente. La población de FB-1, por el contrario, disminuyó de 1.4 x 10^8 a 133 colonias/ml a −20°C y a 2.3 x 10^7 colonias/ml a −80°C, cuando se almacenó durante 150 días.*

Introduction

Ratoon stunting disease (RSD) of sugarcane (*Saccharum* interspecific hybrids) first was discovered during the summer of 1944-1945 in Queensland, Australia (11). The disease is mechanically transmitted on knives and harvesting equipment, and diseased cane had been distributed widely before the disease was discovered in an extremely susceptible variety (9, 11). As a consequence, the disease has been reported in almost every sugarcane producing area of the world.

Since no microorganism could be associated with mechanically transmitted RSD and because sap from infected plants retained its infectivity through extensive dilution, researchers originally suggested that the causal agent was a virus (4, 8, 10, 11). In 1973, a small coryneform bacterium was found associated with RSD (5, 7, 12). The bacterium inhabits xylem vessels of sugarcane (6, 14, 15). The bacteria are curved or straight rods usually measuring 0.25–0.5 x 1–4 μm (5, 13, 15); they undergo septate division and contain mesosomes (13, 14).

In 1980, the RSD bacterium was isolated in axenic culture, and Koch's Postulates were fulfilled (1). After 2 weeks of aerobic incubation on agar at 30°C, colonies were 0.1 to 0.3 mm in diameter, convex, circular with entire margins, and nonpigmented (1). The bacterium is nonmotile and Gram-positive. The RSD bacterium has been named *Clavibacter xyli* subsp. *xyli* (3).

A similar bacterium was isolated from Bermuda grass (*Cynodon dactylon* L. Pers.) by Davis *et al.* (1). This bacterium (but not the RSD bacterium) produces a yellow nondiffusible pigment in culture, and Bermuda grass strains are indistinguishable morphologically and are related immunologically (1). The Bermuda grass bacterium has been named *Clavibacter xyli* subsp. *cynodontis* (3). The ratoon stunting disease and Bermuda grass stunting disease bacteria are related to some known plant pathogenic bacteria which were originally classified in the genus *Corynebacterium* and have been reclassified as *Clavibacter* gen. nov. (3).

The purpose of this study was to obtain information about *Clavibacter xyli* subsp. *xyli* and *C. xyli* subsp. *cynodontis* by determining bacterial growth curves in liquid medium and bacterial longevity *in vitro* at −20°C and −80°C.

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Materials and methods

Strain F-1 of *Clavibacter xyli* subsp. *xyli* and strain FB-1 of *Clavibacter xyli* subsp. *cynodontis* (both from Florida) were grown on SC medium (1) or in RSD broth (3) at 28°C for 8 to 10 days. SC medium contains distilled water, 1000 ml; cornmeal agar, 17 g; papaic digest of soy meal, 8 g; K_2HPO_4, 1 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; 15 ml of a 0.1% (wt/vol) bovine hemin chloride stock solution (deionized water, 200 ml; NaOH, 0.4 g; bovine hemin chloride (Sigma Chemical Co.), 0.2 g; bovine serum albumin fraction 5 (Sigma Chemical Co.) (10 ml of a 20% aqueous solution), 2 g; glucose (1 ml of a 50% aqueous solution), 0.5 g; and cysteine (free base) (Sigma Chemical Co.) (10 ml of a 10% aqueous solution), 1 g. The bovine serum albumin, cysteine, and glucose solutions were filter-sterilized and added to the autoclaved portion at 50°C. The pH was adjusted to 6.6. RSD broth contains deionized water, 1000 ml; yeast extract, 1 g; $(NH_4)_2HPO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 2 g; 15 ml of a 0.1% (wt/vol) bovine hemin chloride stock solution; KCl, 0.2 g; bovine serum albumin fraction 5 (10 ml of a 20% aqueous solution); cysteine (free base), 1 g; glucose (10 ml of a 50% aqueous solution). The bovine serum albumin and glucose solutions were filter-sterilized and added to the autoclaved portion at 50°C. The pH was adjusted to 6.6.

Growth curves. Suspensions of the F-1 and FB-1 strains were prepared in sterilized deionized water and their turbidities measured at 625 nm with a Spectronic 21 spectrophotometer (Bausch and Lomb). The initial concentrations were 5.0×10^6 colony-forming units (cfu)/ml ($A_{625} = 0.15$) for F-1 and 5.0×10^7 cfu/ml ($A_{625} = 0.08$) for FB-1. Three replicate broth cultures of each strain were inoculated by aseptically adding 1 ml portions of the bacterial suspension to 125-ml Erlenmeyer flasks with sidearms containing 25 ml of broth. One flask containing uninoculated broth served as a control. The flasks were incubated without agitation at 28°C. The turbidity of each flask was measured every 24 h for 30 days after inoculation, and the values were plotted against time. Each growth curve study was repeated 3 times, and similar results were obtained each time.

Long-term storage. Turbid suspensions of strains F-1 and FB-1 were prepared in sterilized 0.1 M sodium phosphate buffer, pH 6.8. Each turbid suspension was dispensed into 10 tubes (5 ml/tube). Five tubes were stored at −20°C and five at −80°C. The cfu/ml of the original suspensions were determined by a dilution-plate-count method (1). A tube from each experiment was thawed and the cfu/ml determined every 30 days. The experiment was continued for 120 and 150 days for the F-1 and FB-1 strains, respectively. These experiments were repeated twice, and similar results were obtained both times.

Results

Growth curves. Each point on the growth curves for both bacterial strains in liquid culture (Fig. 1) represents the average turbidity of 3 flasks minus the turbidity of the control flask, which was 0.04. The F-1 strain reached the stationary phase 10 days after inoculation, and thereafter there was a gradual decrease[-1] in turbidity until day 20. The generation time for F-1 was estimated to be 48 h. Rapid growth was observed from day 4 to day 9 for strain FB-1. Thereafter the turbidity changed little, except that between days 12 and 16 there was a consistent slight increase in turbidity indicating a limited resumption of growth. The generation time for FB-1 was estimated to be 24 h. After 30 days of incubation, no cells of either strain grew when transferred to SC agar.

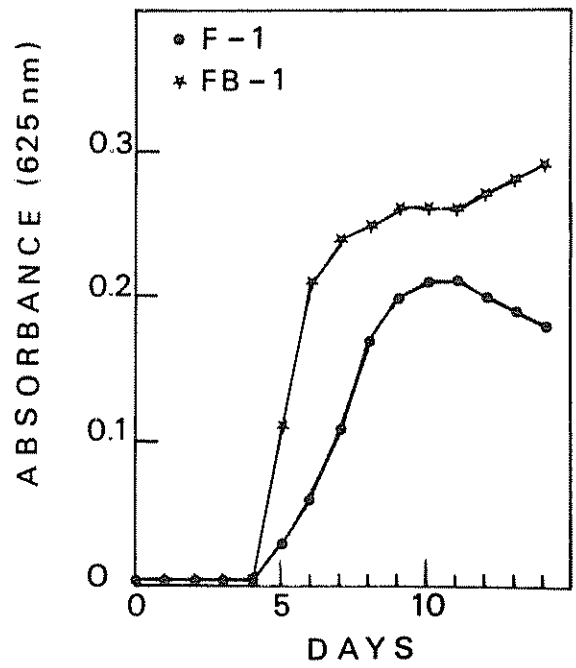

Fig. 1. Growth curves for the F-1 and FB-1 strains of *Clavibacter xyli* spp. *xyli* and *C. xyli* spp. *cynodontis*, respectively. Each point represents the average absorbance values (625 nm) of 3 flasks minus the absorbance of the control flask which was 0.04. The medium was RSD broth. No absorbance was observed in cultures during the first four days.

Long-term storage. During storage at −20°C, the viable F-1 population decreased from 3.5×10^7 to 66 cfu/ml in 120 days. During the same period, but at −80°C, the population of F-1 decreased from 3.5×10^7 to 7.4×10^4 cfu/ml. After the initial 30 day storage time, the shapes of the survival curves for F-1 are similar for both temperatures (Fig. 2). The population of FB-1 decreased from 1.4×10^8

to 133 cfu/ml at −20°C and to 2.3 x 10^7 cfu/ml at −80°C when stored for 150 days (Fig. 2). The survival curves for both strains suggest that a substantial loss of viability occurred during freezing at −20°C, but not at −80°C

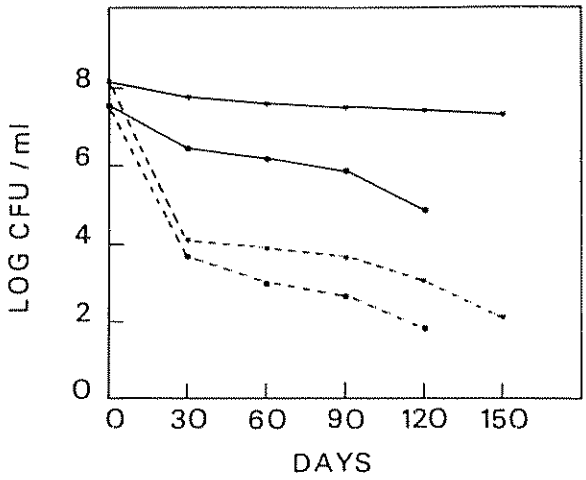

Fig 2. Survival curves for the F-1 (o) and FB-1 (*) strains of *Clavibacter xyli* spp. *xyli* and *C. xyli* spp. *cynodontis*, respectively, when stored at two different temperatures, −20C (– – –) and −80C (—)

Abstract

Growth curves in liquid medium and longevity *in vitro* at −20°C and −80°C were determined for the F-1 strain of *Clavibacter xyli* subsp. *xyli* (ratoon stunting disease) and for the FB-1 strain of *C. xyli* subsp. *cynodontis* (Bermuda grass stunting disease). The generation times were estimated to be 48 h and 24 h for F-1 and FB-1, respectively. The population of viable F-1 cells decreased from 3.5 x 10^7 to only 66 colony-forming units (cfu)/ml at −20°C and to 7.4 x 10^4 cfu/ml at −80°C when stored for 120 days. The population of FB-1 decreased from 1.4 x 10^8 to 133 cfu/ml at −20°C and to 2.3 x10^7 cfu/ml at −80°C when stored for 150 days.

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