Isolation, Culture, and Pathogenicity to Sudan Grass of a Corynebacterium Associated with Ratoon Stunting of Sugarcane and with Bermuda Grass

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ABSTRACT

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The small bacterium associated with ratoon stunting disease (RSD) of sugarcane was successfully cultured in a cellfree medium containing Muller-Hinton agar (3.8%) or Muller-Hinton broth (2.2%), hemoglobin (10 μg/ml), Isovitalex (BBL) (1%), asparagine (0.2%), glutamic acid (0.05%), KH_2PO_4 (0.1%), K_2HPO_4 (0.25%), $MgSO_4 \cdot 7H_2O$ (0.02%), sodium citrate (0.01%), ammonium sulfate (0.05%), $CaCl_2$ (0.5 μg/ml), $ZnSO_4$ (0.01 μg/ml), and $CuSO_4$ (0.01 μg/ml). The same medium was also used to isolate and culture the RSD-like bacterium present in Bermuda grass. The bacterium recovered from either sugarcane or Bermuda grass can infect and multiply in a Sudan grass-sorghum hybrid. Wilting symptoms were observed in some of the inoculated plants. Cultured organisms exhibited lack of motility, absence of endospores, nonacid fastness, and Gram-positive reaction, but often irregular staining, aerobic growth, and a filamentous-rod (0.25–0.35 × 2–5 μm), clublike, or occasionally V-form morphology was observed. Tiny colonies (0.05–0.23 mm in diameter) were observed in agar medium after 8–19 days of incubation at 30 C.

The causal agent of ratoon stunting disease (RSD) of sugarcane was thought to be a virus or a mycoplasma until 1973 when Teakle et al (19), Gillaspie et al (8), and Maramorosch et al (16) reported the presence of a small coryneform bacterium in infected plants. A number of later studies (1,4,6,9–11,20–22) examined the in situ morphology and localization of the organism. All results suggested that the causal bacterium is Gram-positive, filamentous, and exclusively present in the xylem tissues of diseased plants.

The RSD agent has been previously transmitted to or found in a wide variety of graminaceous plants (18) including Johnson grass, sorghum, maize, sweet Sudan grass, and Bermuda grass (*Cynodon dactylon* L.). In Taiwan, a bacterium similar to that observed in ratoon-stunted sugarcane was observed in Bermuda grass displaying the white-leaf and witches'-broom symptoms. Ultrathin sections made by Chen et al (5) revealed the presence of mycoplasmalike organism (MLO) in the phloem and small bacteria in the xylem of the same diseased Bermuda grass plants. The white-leaf and witches'-broom symptoms of the diseased Bermuda grass are presumably the result of MLO infection, but the pathological importance of the bacterium was not known.

This paper describes the method and medium for isolating and culturing the bacterium associated with RSD of sugarcane and Bermuda grass. The pathogenic role of cultured organisms in Sudan grass was demonstrated. Part of the results have been reported (15).

MATERIALS AND METHODS

Plants. Bermuda grass displaying white-leaf and witches'-broom symptoms was collected from a lawn at National Taiwan University (Taipei, Taiwan). Expressed juice from these plants was examined for the presence of short filamentous bacterium under a dark-field microscope. Plants harboring the bacterium were propagated in the greenhouse and later used for the isolation and other studies. The bacteria were never detected in plants propagated from seeds so these were used as controls. Healthy and RSD-infected sugarcane plants were provided by C. T. Chen of the Taiwan Sugar Research Institute (Tainan, Taiwan). The RSD agent also was mechanically transmitted to and maintained in the Sudan grass-sorghum hybrid (NB 280S).

Medium. The medium used in this study was derived from media developed for studying the Legionnaire's disease agent (17) and *Mycobacterium tuberculosis* (13). It contained Muller-Hinton agar (3.8%) or Muller-Hinton broth (2.2%), hemoglobin (10 μg/ml), Isovitalex (1%), asparagine (0.2%), glutamic acid (0.05%), KH_2PO_4 (0.1%), K_2HPO_4 (0.25%), $MgSO_4 \cdot 7H_2O$ (0.02%)' sodium citrate (0.01%), ammonium sulfate (0.05%), $CaCl_2$ (0.5 μg/ml), $ZnSO_4$ (0.01 μg/ml), and $CuSO_4$ (0.01 μg/ml). The Muller-Hinton agar (or broth), hemoglobin, and Isovitalex were purchased from BBL, Division of Becton, Dickinson and Company, Cockeysville, MD 21030. Normally, the Isovitalex and hemoglobin (in 2% stock solution) were added after the other ingredients were autoclaved at 121 C for 10 min and cooled to about 55 C. The final pH of the medium was adjusted to 7.1 ± 0.1 before autoclaving.

Isolation and cultivation. A piece of nodal tissue (approximately 1–2 × 3 mm) excised from diseased sugarcane, Bermuda grass, or Sudan grass was thoroughly washed with tap water and gently blotted with a paper towel. The tissue was surface sterilized by submerging the tissue in 0.1% mercuric chloride or 1% sodium hypochlorite for 1.5–2.0 min. After it was rinsed in sterile water three times, the tissue was comminuted in 0.5–1.0 ml of sterile water or 2.2% Muller-Hinton broth in a petri dish. The sap was expressed from the tissue with forceps and the bacterial suspension was then deposited or streaked onto the agar medium with an inoculating loop. The culture plates were incubated aerobically at 30 C. Bacterial colonies were examined with a dissecting microscope (×20–60). Single colonies were isolated and cloned for further studies. Two isolates were maintained in the agar or broth medium

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and serial passages were made at 1- to 2-wk intervals.

Pathogenicity test. Seeds of the Sudan grass-sorghum hybrid were germinated in a moist chamber. Young plants were transplanted into 15-cm-diameter pots and maintained in the greenhouse. When plants were 3 mo old, two-node cuttings from them were made as previously described (1,2,22). An upright (3) was made by planting a two-node cutting vertically, with the basal bud removed so that the buried node would produce the shoot. For pathogenicity tests, the two-node cuttings were dipped overnight in the bacterial suspension and planted vertically in the pots. Cuttings dipped in the phosphate buffer saline (PBS, 0.02 M sodium phosphate in 0.8% NaCl) were used as controls. For preparation of the bacterial suspension, bacterial cultures on the agar medium were washed and collected in PBS (pH 7.0). The inoculum concentration was approximately 10^7 colony-forming units (cfu) per milliliter or an absorbance of 0.03 at 640 nm. Ten weeks after planting, the sap was expressed from the inoculated uprights and subsequently examined for the presence of bacteria under the dark-field microscope.

Characterization. The cellular morphology of the cultured organisms was examined with a dark-field microscope (Nikon Model M inverted microscope, ×1,500) and with an electron microscope (Siemens A). Negative staining was done with 5% ammonium molybdate. For thin-section observations, the bacterial colonies were fixed with 3% glutaraldehyde and postfixed with OsO_4. The fixed organisms were dehydrated through an ethanol series and then embedded in Spurr's medium (Electron Microscopy Science, Fort Washington, PA 19034). Thin sections (60–90 nm) were cut with a diamond knife and stained with uranyl acetate and lead citrate.

To determine the acid production by the organism, phenol red indicator (25 μg/ml) was added into the culture medium. Acid production by the organism was accompanied by a decrease of the pH and subsequently by a color change in the medium from red to orange or yellow.

To determine the motility of the cultured organisms, a droplet of 10-day-old broth culture was put on a clean slide and examined for motility immediately with the dark-field microscope.

The gas requirement by the cultured organisms was determined as follows: 3 ml of broth medium containing 25 μg of phenol red per milliliter was inoculated with a log-phase culture of bacteria to obtain an initial cell concentration of 10^5 cfu/ml. One-half milliliter of sterile paraffin oil was layered on top of the broth culture. Culture tubes were incubated at 30 C. Color change (from red to orange or yellow) and the bacterial concentration in each tube were determined 3 wk after inoculation.

RESULTS

Although more than 20 media were tested for cultivation of the RSD-associated bacterium, the medium described in Materials and Methods is the only one that was useful. Using this medium in 13 attempts, we were able to isolate and culture the coryneform bacterium repeatedly from sugarcane or Sudan grass infected with RSD. A similar bacterium was also isolated from the Bermuda grass displaying the white-leaf and witches'-broom symptoms. The disease symptoms of Bermuda grass sometimes disappeared completely in the greenhouse, but the bacterium remained abundant in the new shoots. The bacterium was isolated from these symptomless shoots in four of five attempts. Two isolates, RSD-T4 and BG-821, obtained respectively from sugarcane and Bermuda grass were cloned for pathological and biochemical studies.

TABLE 1. Pathogenic tests of cultured organisms on Sudan grass-sorghum hybrid.

Inocula[a]	No. of plants			% Infection[b]
	Inoculated	Infected	Showing wilting	
RSD-T4	39	36	6	92
BG-821	23	5	0	22
Control	28	0	0	0

[a] The inoculum concentration was approximately 10^7 cfu/ml or an absorbance of 0.03 at 640 nm; two-node cuttings dipped in phosphate buffer saline (PBS) were used as controls.

[b] Positive infection was based on the presence of bacteria in inoculated plants after 10-wk incubation.

The pathogenic role of the isolate RSD-T4 and BG-821 in ratoon stunting disease was tested on the Sudan grass-sorghum hybrid. As shown in Table 1, 36 out of the 39 uprights treated with the RSD-T4 bacterial suspension became infected 10 wk after inoculation. The sap expressed from the inoculated plants contained approximately 10^9 bacteria per milliliter. In addition, wilting symptoms especially on the main shoot of the upright were observed in six of 36 infected plants. Neither bacterium nor wilting was found in any control uprights inoculated with PBS. The isolate BG-821 obtained from Bermuda grass also infected and multiplied in the Sudan grass-sorghum hybrid, but no wilting was observed in any uprights infected with the BG-821.

Both RSD-T4 and BG-821 isolates exhibited the characteristics of coryneform bacteria. They possessed a smooth cell wall (Fig. 1C). The Gram-staining was positive, but irregular staining often was observed in aged cells. Observations of cultured organisms under the electron microscope never revealed the presence of endospores and flagella. Mesosomes were always present in the cells examined. Both organisms produced very little acid in the culture medium; it usually took 2 wk or longer to detect a slight color change (from red to orange) or a decrease in pH, but during this time bacterial cell concentration usually reached 10^9/ml. Under anaerobic conditions, no growth was observed with either isolate. No indication of motility was detected under the dark-field microscope. Clublike and filamentous-rod cells were observed in both liquid and agar cultures. Normally, the clublike cells were predominant in the early stage of growth, whereas the filamentous-rod cells were predominant in the later stage. Very frequently the V-form, and golf-club-form cells also were observed (Fig. 1B and D).

Both organisms formed tiny colonies (Fig. 1A) in agar medium after incubation for 8–19 days at 30 C. The size of colonies varied from 0.05 to 0.23 mm (average, 0.17 mm) in diameter. The surface of the colonies was rough and they were readily identifiable by their small size under the dissecting microscope (×20–60). Production of yellowish-orange pigment was found in some isolates obtained from Bermuda grass.

DISCUSSION

Among a number of newly recognized prokaryotes including MLO and RLO (rickettsialike organism), some have been shown to cause plant diseases. Because of the difficulty in the past to culture these organisms, they were termed "fastidious prokaryotes." Recent studies show that some of these organisms can be cultured if suitable media and isolation techniques are applied. For example, with modification in isolation techniques, the corn stunt spiroplasma and *Spiroplasma citri* have been grown in a medium (14) similar to the standard mycoplasma medium. This paper demonstrates that the RSD bacterium can be isolated and cultured in a combination of two media initially developed for the Legionnaire's disease bacterium (17) and *M. tuberculosis* (13), respectively. An independent investigation of Davis et al (7) published while this paper was in review also indicated that the RSD bacterium is cultivable in the cellfree medium. The medium we developed in this work is different from that formulated by Davis et al (7). However, the cellular and growth characteristics of the RSD bacterium reported by Davis et al (7) are similar to those we described earlier (15) and fully accounted here.

Morphologically, the bacterium (isolate RSD-T4) cultured from ratoon-stunted sugarcane resembles that obtained from Bermuda grass (isolate BG-821). Both isolates infect and multiply in the Sudan grass uprights, but RSD-T4 induces higher infection rate and sometimes causes wilt in some of the inoculated plants. Concurrent studies of Davis et al (7) also showed that the Bermuda grass isolate grown in their media neither incites RSD in sugarcane

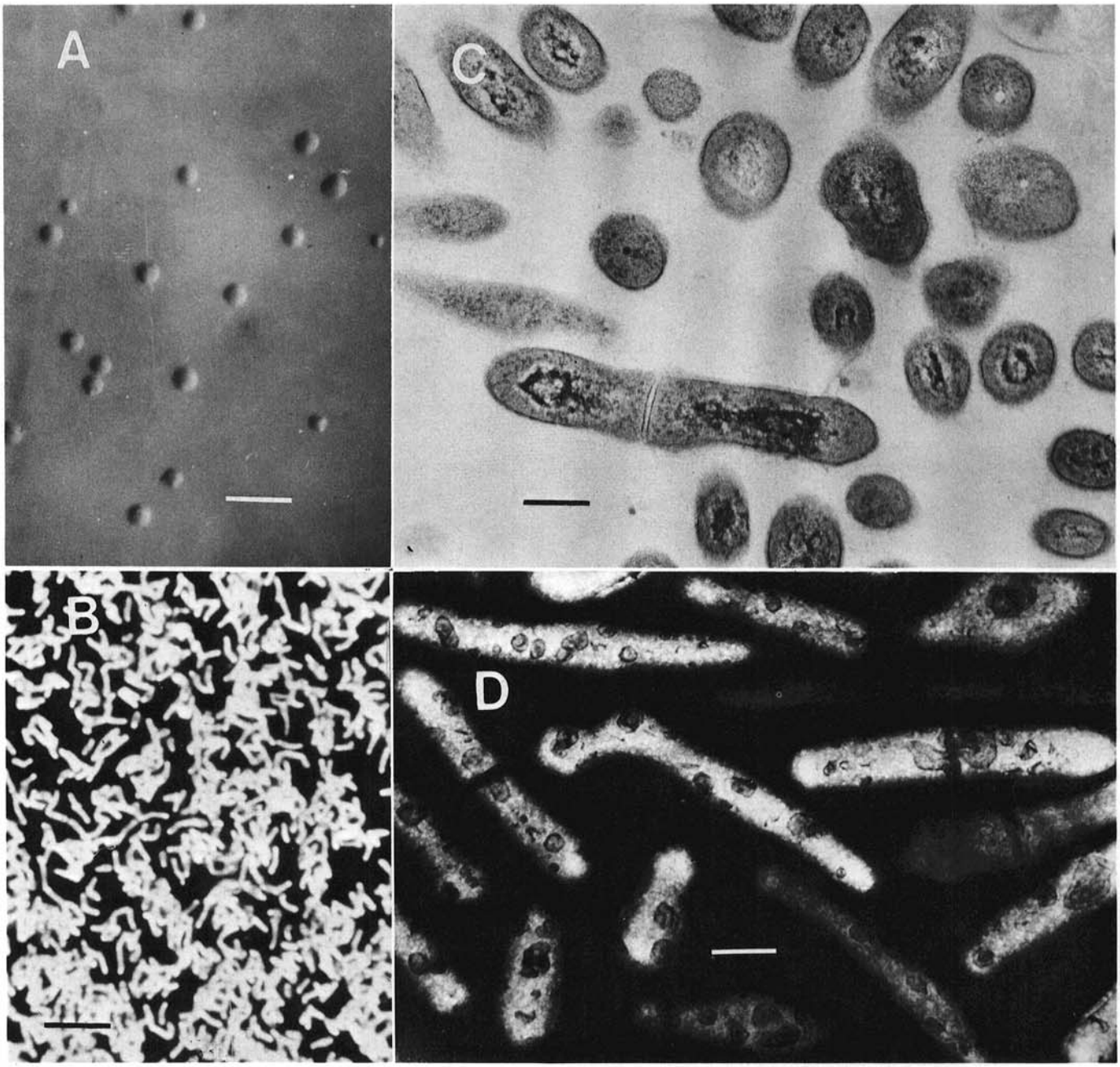

Fig. 1. A, The ratoon stunting agent (isolate RSD-T4) forms tiny, circular, and entire colonies (0.05–0.23 mm in diameter) (bar = 0.4 mm). **B,** The cultured bacteria under the dark-field microscope (×1,500) (bar = 8 μm). Note the pleomorphic nature of the bacterium. **C,** Ultrathin section of the cultured organism (isolate RSD-T4) reveals the presence of smooth cell-wall and mesosomes. Formation of septum is seen in one cell (bar = 0.3 μm). **D,** Negative staining of the cultured organism (RSD-T4) illustrates various forms of cells shown in (B). Mesosomes are always in the cells (bar = 0.3 μm).

nor causes wilting in Sudan grass upright. They suggest that the Bermuda grass isolate represents a different species or pathovar of the RSD bacterium. At present, the pathogenic role of our isolates (RSD-T4 and BG-821) in sugarcane and in Bermuda grass has not been established. The serological, genetic, and biochemical relatedness between these two isolates warrants further examination.

Bermuda grass is a common perennial grass that grows near or in the sugarcane fields in Taiwan. The bacterium similar to that causing RSD was first observed (5) in and isolated from plants exhibiting witches'-broom and white-leaf symptoms. However, the bacterium has also been observed in and isolated from Bermuda grass which was reared in the greenhouse and exhibited no visible symptoms. Hence it is unlikely that the bacterium is responsible for the leaf symptoms and the pathological importance of this xylem-inhabiting bacterium in Bermuda grass is doubtful. On the other hand, in view of our finding that BG-821 can infect Sudan grass, defining its importance as a pathogen to certain sugarcane cultivars will require future investigation.

Based on the in situ formation of microcolonies (10) and the branching of cells (11), Kao and Damann proposed that the RSD bacterium is an actinomycete. We have been unable to confirm the branching under our culture conditions. It is possible that the in vivo morphology of RSD bacterium is somewhat different from that in in vitro conditions. Although all characteristics demonstrated in this report indicate that the RSD bacterium is a coryneform organism (12), we recognize that much work is required before the taxonomic status of the organism can be assigned.

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