

ISOLATION AND CHARACTERISATION OF SOME ENDOPHYTIC BACTERIA FROM PAPUA NEW GUINEA SUGARCANE

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Abstract

Endophytic bacteria were isolated from the stalks of sugarcane, *Saccharum officinarum*, growing in Papua New Guinea. Endophytic bacterial communities from extracted juice averaged 6×10^4 cfu/ml, which is 25 times greater than the number of endophytic bacteria found in commercial varieties in South Africa. Sixty isolates, representing the different colony morphology types, were used for *in vitro* inhibition tests against *Clavibacter michiganensis* subsp. *insidiosus* (a relative of the causative agent of ratoon stunting disease), *Fusarium napiforme*, *F. proliferatum* and *Ustilago scitaminea* (smut). None of the isolates showed inhibition of *Fusarium*. Thirteen belonged to the genus *Burkholderia*, as shown by 16S sequencing; among these, four inhibited the growth of *Ustilago scitaminea* and seven inhibited the growth of *Clavibacter michiganensis*. Six of the *Burkholderia* isolates showed strong chitinase activity.

Keywords: sugarcane, *Saccharum officinarum*, biocontrol, smut, *Clavibacter michiganensis*, *Fusarium* sp., *Burkholderia*, endophyte, Papua New Guinea

Introduction

Endophytic bacteria can actively or latently colonise plants locally or systemically. Various reports indicate that these bacteria exist in a variety of tissue types within numerous plant species, suggesting an ubiquitous existence in most, if not all, higher plants. Endophytes are able to live inside plants without causing disease symptoms (Tervet and Hollis, 1948) and examples of their potential use for agricultural gain have been demonstrated. Their use can be divided into two categories based on types of activity: growth promotion and disease control (Bacon and White, 2000).

Among the bacterial genus, *Burkholderia*, are sugarcane rhizosphere-associated species that are nematode antagonists, plant growth promoters, nitrogen-fixers, and antagonists of fungal and bacterial pathogens (Vogel *et al.*, 2002). In South Africa, few endophytic *Burkholderia* have been isolated from sugarcane stalks and none has had antagonistic properties. This paper reports on the occurrence of several endophytic *Burkholderia*, and numerous other endophytes, in the stalks of ancestral sugarcane from Papua New Guinea, and discusses their biocontrol properties.

Material and methods

The stalks of five different genotypes of the ancestral species, *Saccharum officinarum*, growing in gardens in Papua New Guinea, were used in this study. The stalks varied in thickness, colour and markings.

Culture media

Total endophytic microflora were isolated on PW medium (Atlas, 1997) and Tryptic Soy Broth Agar (Sigma) were used. *Burkholderia* spp. were isolated on a selective medium, PCAT (Burbage and Sasser, 1982). A chitin-containing medium was used to detect chitinase activity (Lingappa and Lockwood, 1962).

Isolation and enumeration

One of each of the stalks of the five *S. officinarum* genotypes was used. From the lowest part of the stalk a three-budded sett was cut and the ends sealed in molten candle wax. Each stalk was washed with tap water and blotted dry. The stalks were then surface sterilised in 10% NaOCl for 10 minutes. The middle internode with its node was removed using sterile secateurs, disinfected with 70% ethanol (avoiding the cut ends) and passed through a flame. The internode was placed in a sterile Petri dish and the core was removed using sterile forceps and a knife. The core was weighed and crushed with a sterile garlic crusher to extract the juice. The volume of juice was measured, serially diluted with 0.8% NaCl, inoculated on the above-mentioned media, incubated at 30°C for 48 hours and assayed for growth. Single colonies on plates were enumerated, described, labeled and streaked for purity on fresh media. The number of bacteria per ml of extracted juice and per gram of plant tissue was calculated.

DNA preparation

Bacterial genomic DNA was extracted by thermal shock. One colony was dispersed in 20 µl of sterile water. Tubes were heated in boiling water for five minutes and placed in the freezer for 10 minutes. Cell debris was centrifuged down and the supernatant used for PCR. Direct PCR was performed on FTA paper¹ containing extracted juice.

PCR amplification and sequencing of the 16S rRNA gene

Burkholderia genus specific PCR (Pallud *et al.*, 2001) and *B. tropica* species specific PCR (Guyon *et al.*, 2003) were performed in 50 µl reaction mixtures containing 2 µl of DNA or (supernatants from colonies and FTA paper), 20 µM of each deoxyribonucleoside triphosphate, 1x PCR buffer, 1.5 mM MgCl₂, 0.5 µM of each primer and 2.5U of Taq DNA polymerase (Promega). Amplification and sequencing of 16S rRNA was followed as in Estrada de los Santos *et al.*, (2001). Identification of species also involved using the V3/H17 region of the 16S rRNA (Balandreau *et al.*, 2004).

Amplified ribosomal DNA restriction analysis

A 5 µl aliquot of each PCR product (16S amplified rRNA) was digested with 1U of the restriction enzymes *AluI* and *HaeIII* (Roche) and 2 µl of corresponding buffer, in a final volume of 20 µl, for three hours at 37°C.

Inhibition tests

Inhibition tests were conducted against *Ustilago scitaminea* (sugarcane smut), *Fusarium napiforme*, *F. proliferatum* and *Clavibacter michiganensis* subsp. *insidiosus*.

¹ Filter Matrix with FTA® Chemistry, Whatman® BioScience Ltd. Abington, Cambridge, UK.

Initially it was intended to use *Leifsonia xyli* subsp *xyli*, the ratoon stunting disease (RSD) bacterium, in the inhibition tests but difficulties were experienced with its culture, presumably due to its obligate parasitic nature. Instead *Clavibacter michiganensis* subsp. *insidiosus*, a close relative, was used. All bacterial strains isolated from the stalks were spot inoculated onto PW medium and incubated for three days at 30°C. The bacteria were killed by chloroform fumigation and washed off with sterile water. After drying for two hours the plates were overlaid with *C. michiganensis* or sporidia of *U. scitaminea* and incubated for 48 hours before scoring them for inhibition zones. For *Fusarium* inhibition assays the bacteria and fungi were plated onto PW medium simultaneously and checked for inhibition zones after three days.

In vitro screening for chitinase activity

Bacterial cultures were streaked on the chitin containing medium and incubated at 30°C for 48 hours. A clearing zone in the medium indicated positive chitinase activity.

Results

Endophytic bacterial communities from extracted juice averaged 6×10^4 cfu/ml, which is 25 times greater than the number of endophytic bacteria found in commercial varieties in South Africa (van Antwerpen *et al.*, 2002). Average numbers were 60 608 bacteria/ml or 43 623 bacteria/g of stalk. Sixty endophytes were characterised according to different colony morphology. Thirteen isolates were identified as species of *Burkholderia*, viz. *B. gladioli*, *B. plantarii* or *glumae* and *B. unamae*. The remaining endophytes were not identified.

Inhibition and in vitro screening tests

All sixty endophytes were subjected to *in vitro* screening. Only nine isolates, all belonging to *Burkholderia*, had properties of interest (Table 1). Isolate NG4J inhibited the growth of smut, while isolates NGJ, NG2 and NG7G inhibited the growth of *C. michiganensis* and were positive for chitinase activity. Isolates NG2B, NG1A and NG3C inhibited the growth of both smut and *C. michiganensis* and were chitinase positive. NG3 inhibited *C. michiganensis* only, while NG1 was chitinase positive. None of the isolates affected the growth of the *Fusarium* species.

Table 1. Characteristics of endophytic *Burkholderia* isolates from *S. officinarum*.

Isolate	<i>Fusarium napiforme</i>	<i>Fusarium proliferatum</i>	<i>Clavibacter michiganensis</i>	Chitinase activity	<i>Ustilago scitaminea</i>
NGJ	-	-	++	++	-
NG2	-	-	++	++	
NG7G	-	-	++	++	
NG2B	-	-	++	++	+
NG1A	-	-	++	++	+
NG3	-	-	++	-	-
NG1	-	-	-	+	-
NG3C	-	-	++	++	++
NG4J	-	-	-	-	+

+ Slow inhibition/activity \geq 3days; ++ Rapid inhibition/activity \leq 3days; - No effect.

Discussion

Sugarcane is indigenous to Papua New Guinea, with several species of *Saccharum* and hundreds of cultivars present (Kuniata *et al.*, 2001). Wild sugarcane has been challenged for millions of years by pests and diseases. Beneficial endophytes might have participated in the evolutionary success of *Saccharum* spp. through their growth promotion and biocontrol properties.

Burkholderia species isolated from Papua New Guinea colonised plant tissues without producing obvious effects on the plant. However, they only represent a small percentage of the total culturable endophytic community, suggesting that they are not true endophytes. *Burkholderia* spp. normally associate with the plant rhizosphere, and under appropriate conditions can invade and colonise internal tissues. In this case, the isolated *Burkholderia* spp. are opportunistic endophytes that are capable of utilising organic compounds within sugarcane for growth and survival. They can withstand the physiological conditions encountered within the plant's tissues, suppress pathogen infection of the host and avoid induction of host defense responses that could eliminate them.

Six of the endophytic *Burkholderia* isolates produced chitinase and inhibited the growth of *C. michiganensis*. The production of chitinase might be exploited for the control of *Meloidogyne* species, and other nematodes, as nematode eggshells are degraded by this enzyme (Mercer *et al.*, 1992).

The main method of controlling disease in the sugar industry is through the use of resistant varieties. However, in the case of RSD virtually none of the South African varieties possesses adequate resistance or tolerance and the control of this disease depends entirely on cultural practices. Endophytic *Burkholderia* isolates could be developed as biocontrol agents for the control of RSD and smut.

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