ISOLATION AND IDENTIFICATION OF N₂-FIXING ENTEROBACTERIA ASSOCIATED WITH SUGARCANE IN TROPICAL AUSTRALIA

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The nitrogenase activities of enterobacteria isolated from sugarcane roots and sugarcane waste products were usually 100–400 nmol C₂H₄/culture/h. The isolates were characterized both phenotypically and chromosomally. Four phenotypic groups were differentiated. Group 1 isolates were identified as *Klebsiella pneumoniae* by computer-aided Microbact System 24 E and showed over 70% DNA homology with *Klebsiella pneumoniae* UQM 90. Isolates of group 3 and group 4 were identified as *Enterobacter cloacae* and *Enterobacter agglomerans* respectively by the Microbact 24 E, but DNA-DNA hybridization studies suggested that isolates of these 2 groups belonged to the same species and did not belong to either *Enterobacter cloacae* or *Enterobacter agglomerans*. Group 2 isolates were identified as *Enterobacter agglomerans* by the Microbact 24 E, but G + C% and DNA-DNA hybridization studies contradicted this identification. The G + C% values and phenotypic characteristics indicated that group 2 isolates might represent a new species of the genus *Klebsiella* while the isolates of group 3 and group 4 together could constitute a new species of the genus *Enterobacter*.

Enterobacteria were the dominant N₂-fixing bacteria isolated from sugarcane roots (19). There were also reports of the isolation of N₂-fixing enterobacteria from the rhizosphere of rice (2, 10, 22), wheat and sorghum (18), grasses (5, 17, 24), and dicotyledonous plants (4), as well as from grassland soil (12), bark of a mangrove tree (23) and paper mill process water (16). The isolated N₂-fixing enterobacteria were generally identified to the species level using selected biochemical tests or computer-aided commercial microtube systems. No chromosomal studies of these N₂-fixing enterobacteria were attempted, although base composition of chromosomal deoxyribonucleic acid (DNA) of microorganisms and degree of DNA

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homology between two microorganisms have proved to be extremely useful in the classification and identification of microorganisms over the past 20 years. We report here (a) the isolation of N_2 -fixing enterobacteria from sugarcane roots and sugarcane waste products and (b) the classification and identification of isolates by DNA base composition analyses and DNA-DNA homology studies in addition to computer-aided Microbact System 24 E.

MATERIALS AND METHODS

Isolation of N_2 -fixing enterobacteria. Whole root systems of sugarcane plants were carefully removed with attached surrounding soil from sugarcane fields in Queensland, Australia and transported to the laboratory in plastic bags. Variety Q137 second ratoon plants were collected from alluvial soils of the Bureau of Sugar Experiment Stations farm at Eight Mile Plains, Brisbane (28°S and 153°E). Variety Q110 second ratoon plants and variety CP44-101 fourth ratoon plants were sampled from alluvial soils of farmers' fields at Rocky Point, approximately 60 km south of Brisbane, which had not received N-fertilizer for the previous 2 years. Variety CP44-101 fourth ratoon plants were also sampled from adjacent fields that had been fertilized. Variety CP44-101 plant cane and first ratoon plants were sampled from farmers' fields near Nambour (Clay soil, 26.6°S and 153°E). Sugarcane trash and bagasse were sampled from Mackay (21.5°S and 148°E). Trash samples were collected in sugarcane fields of a peat loam soil, in which the trash had been left and undergone decomposition for 12 months at time of sampling. Trash samples were also collected in fields of a sandy loam soil, in which the trash had been left and undergone decomposition for 6 months at time of sampling. Bagasse samples were obtained from about 10 cm inside bagasse heaps which had been stored for 3 months. Samples were treated for bacterial isolation immediately upon reaching the laboratory.

Roots were separated from the setts and shoots and shaken gently to remove loosely attached soil, after which they were washed in phosphate-buffered saline (PBS, pH 7.2) with gentle agitation. The soil slurry obtained was taken as the rhizosphere soil. Microorganisms on the root surface (rhizoplane section) were released by vigorously shaking the roots in PBS with glass beads. Roots were subsequently surface-sterilized with 96% (vol/vol) ethanol for 10 min and washed 3 times with PBS. After surface sterilization the roots were cut into small pieces which were then ground to a slurry (endorrhizosphere section) in PBS using a sterile mortar and pestle. Sugarcane trash and bagasse were also ground to slurries for inoculation of isolation media.

Each synthetic N-free medium (SNFM) contained the same basal salt components of the combined carbon medium (19) but was amended with carbon sources as indicated. Each semisolid SNFM contained 0.2% agar and was supplemented with 0.002% yeast extract, whereas each solid SNFM contained 1.5% agar and was supplemented with 0.01% yeast extract. The pH for all media was 7. All

enrichment, isolation and characterization cultures were incubated at 28°C except as otherwise indicated.

Serum bottles (13 ml) containing 5 ml semisolid SNFM with either 1.5% sucrose, mannitol, malate, glucose or a combination of each of 0.5% sucrose, 0.5% mannitol and 0.5% malate were inoculated with appropriate dilutions of rhizosphere soil, rhizoplane or endorhizosphere sections of the roots or small root pieces. After incubation for 2 to 3 days the acetylene reduction assay (ARA) was performed to detect the presence of nitrogenase activity of each enrichment culture as described previously (11). Any ARA positive cultures were subcultured 3 times before being streaked onto solid SNFM containing the same carbon substrates as the semisolid SNFM and incubated under air for 2 to 3 days. Isolation was also attempted from some of the roots as well as from the trash and bagasse samples by directly spreading the appropriate dilutions of homogenized slurries onto the solid SNFM plates without pre-enrichment in semisolid SNFM. These cultures were subsequently incubated under air, in glove bags flushed with pure nitrogen or anaerobically using a BBL Gas Pak anaerobic system until visible growth was observed.

Different types of single, well-separated colonies which had grown on solid SNFM were picked and transferred into semisolid SNFM to check the acetylene reduction activity. Final isolation and purification were done using nutrient agar (NA). As our isolates were identified as *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Enterobacter agglomerans* by the Microbact System 24 E (Disposable Products Pty Ltd, Adelaide, South Australia), *E. cloacae* UQM 1975^T, *E. agglomerans* UQM 1615 and *K. pneumoniae* UQM 90 as well as *Escherichia coli* UQM 1803^T were included as reference cultures. These reference cultures were obtained from the Culture Collection, Department of Microbiology, The University of Queensland. All cultures were maintained on NA slants at 4°C and transferred monthly. Before use, all cultures were checked for purity on NA. One-day-old single colonies grown on NA were used as inocula or used directly for characterization studies.

Nitrogenase activity of pure isolates. Faintly visible bacterial suspensions were obtained by suspending single colonies of the isolates in PBS; these bacterial suspensions were gently shaken into 5 ml of melted (45–50°C) semisolid mannitol SNFM in 13 ml serum bottles. After incubation at 28°C for 16–22 h, the nitrogenase activity of each culture was determined by the ARA.

Phenotypic characteristics. The Gram reaction, cell morphology and motility were determined according to Skerman (21). Presence and arrangement of flagella of motile isolates were determined by electron microscopy using cell preparations negatively-stained with uranyl acetate-sucrose. Oxidation or fermentation of glucose was determined by the method of Hayward (6). Oxidase reaction was determined using Oxidase Detection Strips (Disposable Products Pty Ltd, Adelaide, South Australia). Pigmentation was determined on GPYE (1% glucose, 1% peptone and 0.5% yeast extract) agar and solid sucrose SNFM which was supplemented with 0.2% yeast extract and 0.2% NH₄Cl. Enterobacteria isolates were

further characterized by the Microbact System 24E and species identification of bacteria was achieved through the Microbact Computer Aided Identification Package Version 1.1 System 3 (Disposable Products Pty Ltd, Adelaide, South Australia).

DNA extraction and purification. Flasks (250 ml) containing 100 ml of liquid GPYE (1% glucose, 1% peptone and 0.5% yeast extract) medium were inoculated with selected isolates or the reference cultures and incubated at 28°C with shaking for 16–22 h. Cells were harvested by centrifugation at $10,000 \times g$ at 4°C for 10 min. The cell pellets were washed by resuspending in cold saline-EDTA (0.15 M NaCl, 0.1 M EDTA disodium salt, pH 8) at 4°C and cells were harvested by centrifugation at $10,000 \times g$ at 4°C. This washing procedure was repeated for 3 times. DNA was extracted and purified by the method of Marmur (14) with some minor modification.

DNA base composition. The DNA base composition was determined by the thermal denaturation method of Marmur and Doty (15). Purified DNA samples were diluted to about 0.025–0.030 mg/ml with $0.1 \times \text{SSC}$ (absorbance 0.5–0.6 at 260 nm). The analyses were carried out at 260 nm in a Gilford 2600 UV microprocessor-controlled spectrophotometer linked to a thermoprogrammer which was set to increase the temperature of the DNA samples at 0.5°C per min. The change in absorbance of the sample was recorded with a Hewlett-Packard plotter. The DNA base composition (mol% guanine plus cytosine) was calculated by using the modified formula of Mandel and Marmur (13) with T_m values replaced by T_i values which were obtained from second derivative plots (20), and with *E. coli* UQM 1803 (51.7 mol% G + C) as an internal reference standard.

DNA homology studies. Purified DNA samples were diluted to about 0.05 mg/ml (absorbance about 1.0 at 260 nm) with $0.1 \times \text{SSC}$ and the DNA fragmented by sonication of 2 ml samples for 20 s using a Branson Sonifier 250 equipped with a microtip with duty cycle constant and an output approaching 40 units. Sheared DNA samples were dialyzed against 3 changes of $2 \times \text{SSC}$ overnight. Sheared DNA samples from 2 individual bacterial strains were adjusted to the same absorbance (0.8–1.0 at 260 nm) in $2 \times \text{SSC}$ and equal volumes mixed. Homologous and heterologous DNA samples in 1-cm path quartz microcuvettes were denatured by heating to 99°C. Renaturation kinetics in $2 \times \text{SSC}$ at 260 nm were analyzed and recorded with the same equipment as for the DNA base composition determination. Absorbance decreases for the 2 individual strains or a mixture of the 2 from 5 to 25 min, 10–30 min and 15 to 35 min were measured from the slopes of the renaturation plots and used to calculate the degree of binding (D) according to the equation of Huss et al. (7).

RESULTS

Isolation of N_2 -fixing enterobacteria

Among the isolates obtained, 49 isolates were found to be Gram-negative,

facultatively anaerobic, oxidase negative and rod-shaped, and proved to be enterobacteria. These isolates produced acid and gas, and developed C₂H₂ reduction activity in semisolid SNFM with either 1.5% sucrose, 1.5% glucose or 1.5% mannitol as carbon source after incubation for 1–2 days. With mannitol as C source, the nitrogenase activities of these isolates were usually 100–400 nmol/culture/h (Table 1), which were comparable to the nitrogenase activities of *Azospirillum* or *Herbaspirillum* (1). Growth developed throughout the semisolid medium without the formation of surface or subsurface pellicles. All of the motile isolates had peritrichous flagella. Some phenotypic characteristics of the isolates and identifications obtained from Microbact 24 E are presented in Table 1. The isolates were differentiated into 4 groups. Fourteen of the nonmotile isolates which did not produce pigment, and which were identified as *Klebsiella pneumoniae* by the Microbact System 24 E, were assigned to group 1. The remaining 6 nonmotile isolates identified as *Enterobacter agglomerans* by the Microbact 24 E produced diffusible brown pigments on GPYE agar after aging, and were assigned to group 2. Group 3 consisted of the 16 motile isolates which were identified as *Enterobacter cloacae*. These isolates produced non-diffusible light yellow pigments on solid sucrose SNFM supplemented with 0.2% yeast extract and 0.2% NH₄Cl. Group 4 contained the motile isolates which did not produce pigments, and were identified as *Enterobacter agglomerans*.

DNA base composition

The G+C% values for the selected isolates are summarized in Table 1. The G+C% values were 58–59% for isolates of group 1, 57–58% for group 2, 54–55% for group 3 and 52–53% for group 4. The G+C% values obtained for the authentic cultures were *Klebsiella pneumoniae* UQM 90, 58.3%, *Enterobacter cloacae* UQM 1975, 55.4% and *Enterobacter agglomerans* UQM 1615, 57.7%.

DNA homology studies

The results of DNA hybridization between isolates and authentic reference cultures are presented in Table 2. Group 1 isolates shared over 70% DNA homology with *Klebsiella pneumoniae* UQM 90. The levels of DNA homology for isolates of the other 3 groups with any one of the reference cultures were 21–34%. The results of DNA-DNA hybridization between isolates within phenotypic groups 2, 3 and 4 are also given in Table 1. DNA homology values among isolates within the same phenotypic group were generally above 90%. The results of DNA hybridization experiments between isolates belonging to different phenotypic groups are presented in Table 3. Isolates of group 3 shared 67–78% homology with isolates of group 4. Other intergroup homology values were generally below 30%.

DISCUSSION

Studies by Johnson (8) have shown that in the DNA homology range

Table 1. Phenotypic characteristics of N₂-fixing enterobacteria isolated from sugarcane roots and sugarcane waste products in Queensland.

Character	Group 1 (14) ^a	Group 2 (6)	Group 3 (16)	Group 4 (13)
Microbact ID	<i>Klebsiella pneumoniae</i>	<i>Enterobacter agglomerans</i>	<i>Enterobacter cloacae</i>	<i>Enterobacter agglomerans</i>
Nitrogenase ^b	87–256	111–390	110–198	58–182
Motility	—	—	+	+
Pigments				
on GPYE	—	+	—	—
on SNFM	—	—	+	—
Acid from				
Glucose	+	+	+	+
Mannitol	+	+	+	+
Xylose	+	+	+	+
Arabinose	+	+	+	+
Inositol	+	+	—	8%
Sorbitol	+	—	88%	92%
Rhamnose	+	—	+	+
Lactose	+	+	+	54%
Raffinose	+	—	—	—
Salicin	+	—	63%	+
Adonitol	—	—	6%	—
Utilization of				
Citrate	+	+	+	+
Malonate	+	+	+	+
Decarboxylase				
Lysine	+	+	+	+
Ornithine	—	—	—	—
Dihydrolase				
Arginine	—	+	+	46%
H ₂ S	—	—	—	—
O.N.P.G.	+	+	+	+
Indole	—	+	—	—
Urea	+	+	+	+
VP	+	—	+	92%
TDA	—	+	81%	+
Gelatin	—	—	25%	46%
G + C%	58–59%	57–58%	54–55%	52–53%
	(6) ^c	(5)	(9)	(10)
Intragroup DNA homology	ND	98–100%	87–102%	93–102%

Note: “+” indicates positive, “—” indicates negative, figures indicate percentage positive.

^a Number of isolates obtained.

^b Nitrogenase activity expressed as nmol C₂H₄/culture/h.

^c Number of isolates tested.

80–100% the major variations that are being measured are differences in the genome size and the alterations in the linear arrangements of cistrons, whereas in the homology range of 30–50%, differences in the base sequences of the cistrons

Table 2. DNA homology values between N₂-fixing enterobacteria isolates and reference cultures.

	<i>Klebsiella pneumoniae</i> UQM 90	<i>Enterobacter cloacae</i> UQM 1975	<i>Enterobacter agglomerans</i> UQM 1615
Homology (%)			
Group 1	74-79 (6) ^a	26 (1)	23 (1)
Group 2	22-34 (5)	23 (1)	26 (1)
Group 3	21 (1)	21-25 (4)	28-29 (4)
Group 4	24 (1)	18 (1)	21-23 (2)

^a Number of isolates tested.Table 3. DNA homology values between N₂-fixing isolates belonging to different phenotypic groups.

	Group 1	Group 2	Group 3	Group 4
DNA homology value (%)				
Group 1	—	25-32 (2) ^a	23 (1)	30 (1)
Group 2	—	—	26 (1)	24 (1)
Group 3	—	—	—	67-78 (3)

^a Number of pairs of isolates studied.

become increasingly important. DNA homology values of 70% have been considered to represent a sufficient degree of genetic relatedness as to warrant consideration of the strains as belonging to a single species (3,8). Consequently, isolates of group 1 would appear to belong to *Klebsiella pneumoniae*. The Microbact identification was in agreement with chromosomal studies for this group. The DNA homology experiments also indicated that isolates of group 3 and group 4 should belong to the same species as intergroup homology values were about 70% or higher, although they were identified as *Enterobacter cloacae* and *Enterobacter agglomerans* separately by the Microbact 24 E. On the other hand, although group 2 and group 4 were both identified as *Enterobacter agglomerans*, it is evident that these two groups are not closely related as there are significant differences in their G+C% contents. In fact, group 2 isolates are believed to be more related to the *Klebsiella pneumoniae* isolates (group 1) as the G+C% values of this group are identical to those of group 1, but different from the others. Also, isolates of group 2 were nonmotile, an important character of the genus *Klebsiella*. Isolates of groups 2, 3 and 4 showed DNA homology with the three reference culture below

30%. Therefore isolates of these groups do not belong to any of these species. Therefore the Microbact identification of our isolates only partially agreed with the chromosomal studies.

From the chromosomal data, it is evident that group 3 plus group 4 and group 2 constitute two separate homologous unidentified taxa. DNA homology studies were only considered most useful for identification at the species level rather than the genus level, and a lack of significant DNA homology would not preclude the assignment of an organism to a particular genus (9). It is likely that ribosomal ribonucleic acid-DNA (rRNA-DNA) studies would be useful at the genus level, since they indicate broader relationships than were evidenced from DNA-DNA studies. In this study, the phenotypic characteristics as well as the mol% G+C values of cellular DNA are considered appropriate in the absence of any DNA-rRNA data. The isolates of group 3 plus group 4 might constitute a new species of the genus *Enterobacter* based on their biochemical characteristics and G+C% content. Isolates of group 2 should be assigned to the genus *Klebsiella* based on the absence of motility and G+C% content.

N₂-fixing enterobacteria isolated from various ecosystems were identified by conventional biochemical tests or with commercially available microtube systems as *Enterobacter cloacae* (10, 16–19, 22–24), *Enterobacter agglomerans* (5), *Klebsiella pneumoniae* (5, 12, 18, 19, 24) or other species (10, 12, 16, 18, 19, 22, 23). Except for the *Klebsiella* isolates, our isolates were wrongly identified by the Microbact System 24E. It seems that care should be exercised when using commercial microtube systems to identify environmental isolates, and in this particular case species of N₂-fixing enterobacteria.

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