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Identification of carbohydrate degrading bacteria in sub-tropical regions

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Abstract: Several bacteria from the islands of Puerto Rico, Vieques and Trinidad were isolated for their carbohydrate degrading activities. These terrestrial and marine bacterium were collected from pineapple agricultural lands, tropical rain forests, coastlands and mangrove swamps. Organisms were screened for activity using chromogenic substrates (AZCL® Megazyme International Ltd., Ireland).The media composition for the effective culturing of some of the marine organisms has also been standardized. Gram-negative organisms were identified by sequence analysis of the PCR- amplified partial small subunit rRNA gene. Results indicate that the majority of the marine organisms tested belong to the genera *Vibrio* and *Pseudoalteromonas* and in the terrestrial environments *Chryseobacterium* predominated. These experiments reveal that sub-tropical environments are potentially good sources of microorganisms with novel carbohydrase activities.

Key words: Caribbean, sub-tropical, carbohydrates, PCR, 16S rRNA gene, AZCL® substrates.

Carbohydrates, the most abundant molecules in nature are used by most organisms as a structural and/ or storage compounds. Carbohydrates over the centuries have been largely used as sources of energy, food and medicines. They are produced in plants, bacteria and marine animals (Teeri 1997). Among the carbohydrates the most abundant are cellulose, starch and xylans, which also represent the largest renewable source of energy known.

Carbohydrases, enzymes that break down carbohydrates into simple sugars, are important in life processes and industrial applications. Acting in a singular or synergistic manner they permit the use of low value bio-mass for the industrial production of paper, textiles, chemicals, feed stuff, bio-fuels and substitutes for currently used toxic substances (Gilbert and Hazlewood 1991). Microbes play an important role in the production of industrial carbohydrases, which represent 28% of total enzymes produced (Rao *et al*. 1998). Functionally complete cellulase enzyme systems, important factor for the conversion of low value bio-mass to ethanol by fermentation (Himmel *et al*. 1997), can be produced by a large diversity of microorganisms, such as aerobic and anaerobic bacteria, white rot fungi and anaerobic fungi (Kubicek *et al*. 1993). Because of their rapid growth rate and relative ease of cultivation, carbohydrolytic microbes have gained significance in recent years. The most characterized and studied of the cellulase systems is that of the rot fungus *Trichoderma ressei* (Kubicek *et al*. 1993). *T. ressei* mutants are generally recognized to be the best strains currently available for the industrial production of cellulases (Kubicek *et al.* 1993). *Clostridium thermocellum* an anaerobic thermophilic bacterium was discovered to have a

TABLE 1

Collection sites

A	Mangrove channels and swamps, La Parguera, Southwest Puerto Rico.
B	River Canyon central mountain range, Puerto Rico.
C	Thermal hot springs, Coamo, south central coast, Puerto Rico.
D	Salt marsh, Southeast Vieques island, Puerto Rico.
E	Mangrove channels, Trinidad and Tobago.
F	Agricultural soil, Lajas, southwest Puerto Rico.
G	Tropical rain forest, Yunque, northeast Puerto Rico.

potential for native crystalline cellulose degradation, which is 50 times higher than other microbial systems (Johnson *et al*. 1982).

Considering that less than 1% of extant bacteria, marine and terrestrial have been isolated and described and an even smaller fraction has been examined for its biodegradation ability (Tiejde 1994), enzymes with improved properties can potentially be isolated. The tropical rain and mangrove forests with their abundance in plant litter provide an ideal stable environment to isolate microorganisms that show improved polysaccharide degrading abilities. In this research, some of the biodiverse regions of the Caribbean were screened for bacteria with different carbohydrase activities. The potential carbohydrase producers were identified by partial 16S rRNA gene sequencing.

MATERIAL AND METHODS

Sample collection and bacterial isolation: The sample collection sites are from different habitats within the Caribbean belt (Table 1) i.e. mangrove swamps and forests, coastlands, agricultural lands, tropical rain forest, hot spring and mountain river canyon.

Soil, plant litter and water samples (3-4 samples per site) were collected in sterile 50 ml polypropylene tubes. These were immediately transported to the laboratory. 10% soil and plant litter suspension with sterile distilled water or sterile sea water were prepared and 0.1 ml of the sample suspension was plated out on solid rich nutrient medium Luria Agar or Bacto®Marine Agar at pH 7.6 or 4.6 at room temperature (23-25°C) for three-seven days. 0.1 ml of the water samples was plated out directly on to the plates. Individual colonies were isolated from these plates and inoculated on seven different azurin insoluble crossed linked chromogenic carbon substrates (AZCL®, Megazyme, Ireland Ltd., 1% w\v) plates at pH 4.6 and 7.0 respectively. Carbohydrase activity was observed by blue dye release from the chromogenic substrates. Growth curves on some of the possible cellulose degraders were accomplished in artificial sea water (Burkholder 1963), insoluble 1% laboratory grade cellulose supplemented with 0.01% yeast extract, 0.1% NH_4Cl, 0.025% Hepes at pH 7. Plate count method was used for the enumeration (see discussion).

Bacterial DNA extraction: Bacterial genomic DNA was isolated using a modification of existing techniques (Ausubel *et al.* 1995). Briefly the technique entailed the following: 5ml of mid- to late log phase bacteria were collected by centrifugation and resuspended in a small volume of lysis buffer (0.05M Tris HCL pH 8.0, 0.01M Na_2EDTA). SDS was added to this solution at a final concentration of 0.5%, mixed gently and incubated for five minutes at room temperature. The solution was sequentially washed with equal volumes of TE (pH 8.0) saturated phenol, phenol-chloroform and chloroform. DNA was precipitated by adding 1/10 volume of 1M NaCl and 2.2 volumes of 100% ethanol and incubated for one hr. at 20°C. The precipitated DNA, after centrifugation was rinsed three times with 70% ethanol, dried and resuspended in sterile distilled water.

Polymerase chain reaction: The genomic DNAs of gram-negative bacterial samples were used for PCR amplification. Approximately 500 base pairs of the 5'end of the 16S rRNA gene was amplified using highly conserved primers $BSF8_{(S)}$ 5'- AGAGTTTGATCCTGGCTCAG-3'(starting at nucleotide 8 on the *Escherichia coli* numbering system) and $BSR538_{(AS)}$ 5'ATTACCGCGGCTGCTGGC-3'(nucleotide 538 on the *E. coli* numbering

system). The amplification conditions were as follows: 30 amplification cycles with denaturing at 95°C for one minute, annealing at 35°C for one minute and extension at 72°C for one minute. Amplified PCR products were purified by passage through Centri-Sep™ spin columns (Princeton Separations Inc.). Both strands of the amplified 16S rDNA templates were sequenced using ABI Prism® Big Dye™ Terminator reaction and the ABI 310 genetic analyzer. The DNA sequence data were analyzed using BLASTN (Altschul *et al*. 1990).

RESULTS

Tropical environments represent high biodiversity due to their stable temperatures, humid climate and a wealth of different habitats. These characters make them one of the most favorable environments for the isolation of microorganisms with improved and stable enzyme activities. In this study we present an initial attempt to isolate bacteria with carbohydrase activities from the Caribbean region.

Initial screening resulted in the isolation of 13 bacterial isolates with carbohydrate degrading abilities. Seven of these were marine in origin (Table 2). These marine isolates showed degradation of amylose, pullulan and cellulose but could not degrade galactomannanan, xylan and arabinan. The terrestrial isolates degraded amylose and to a lesser extent pullulan and arabinan but could not degrade cellulose (Table 2). Our screening did not result in the isolation of dextran degraders. A majority of the bacteria showed degradative abilities at acidic as well as neutral pH.

Colonies isolated were initially separated on the basis of gram staining. Results showed that 12 of the 13 species tested were gram-negative (#156 was gram variable). DNA was isolated from the gram-negative bacteria and 540 base pairs of the 5' end of the small subunit ribosomal RNA gene was amplified utilizing conserved primers (see materials and methods). The isolated partial ssu rRNA gene was sequenced and the generated sequences were compared to those in the GenBank database using the BLASTN program (Altschul *et al*. 1990). Sequence homology of greater than 99% indicates a species match (Stackebrandt and Goebel 1994). Results indicate that *Pseudoalteromonas* is the most predominant genus with carbohydrate degrading abilities found in the marine environments while in the terrestrial environment, *Chryseobacterium* predominates (Table 3). Attempts are now underway to identify strain #156.

The potential for any organism as an enzyme producer depends upon its growth ability in liquid culture. Since less is known about culturing marine organisms as compared to their terrestrial counterparts (Oliver 1993), attempts were made to cultivate some of our marine isolates (#101, #119 and #121) in a well defined medium. Growth on a medium containing artificial seawater (Burkholder 1963), ammonium chloride and microcrystalline cellulose resulted in limited growth with cells reaching static phase in five days. The cells reached a population density of approximately 10^8 colony forming units (cfu)/ml of medium with a generation time of eight hours (data not presented). When the above medium was supplemented with 0.01% yeast extract as a source of vitamins static phase was reached in three days with a cell density of 10^{12} cfu/ml and a generation time of approximately four hours (data not presented).

DISCUSSION

This research is an initial survey of bacteria from the Caribbean region with unusual carbohydrate degrading enzymes with improved properties. The possibilities of isolating such enzymes from tropical environments are relatively high (Tiedje 1994) due to richness of biodiversity there.

Most of the samples collected had very high densities of culturable bacteria (10^5—10^7 cfu/ml), but the numbers of carbohydrate degraders isolated were very small (2-6% of total isolates). The total culturable bacteria from marine sources was approximately an order of magnitude greater than their terrestrial counterparts (data not shown). This indicated that in both coastal marine as well as terrestrial tropical environments tested, the presence of accessible carbohydrates may be relatively

TABLE 2

Carbohydrate degrading bacteria on insoluble chromogenic substrates(AZCL) at pH 4.7 and 7.0

ID#	Azurine-crosslinked polysaccaride insoluble chromogenic substrates													
	Amylose		Pullulan		Cellulose		Dextran		Galactomannan		Arabinan		Xylan	
	pH 4.7	pH 7.0	pH 4.7	pH 7.0	pH 4.7	pH 7.0	pH 4.7	pH 7.0	pH 4.7	pH 7.0	pH 4.7	pH 7.0	pH 4.7	pH 7.0
101	+	+	+	+	+	+	-	-	-	-	-	-	-	-
108	-	-	-	-	-	-	-	-	+	-	-			
119	+	+	+	+	+	+	-	-	-	-	-	+	-	-
121	+	+	+	+	-	+	-	-	-	-	+			
155	+	+	+	+	-	-	-	-	-	-	-	-	-	-
156	+	+	+	+	+	+	-	-	-	-	-	-	-	-
165	+	-	+	-	-	-	-	-	-	-	-	-	-	-
168	-	+	-	-	-	-	-	-	-	-	-	-	-	-
169	+	+	-	-	-	-	-	-	+	+	+	+	+	+
171	+	+	-	-	-	-	-	-	+	+	+	+	+	+
172	-	-	-	-	-	-	-	-	-	-	+	-	-	-
182	+	+	+	+	-	-	-	-	-	-	-	-	-	-
201	+	-	-	+	-	-	-	-	-	-	-	-	-	-

TABLE 3

Sample identification and collection site. Percentage match based on BLASTN searches

Code#	Genus	Collection site	Colony characteristics
101M	*Pseudoalteromonas* sp. (98.5%)	A	White
108M	*Riemerella* sp. (95%)	A	Yellow
119M	*Pseudoalteromonas* sp. (97%)	A	Yellow
121M	*Vibrio* sp. (98%)	A	White
155M	*Pseudoalteromonas* sp. (97%)	E	Orange
156M	Unknown sp.	D	Reddish
165T	*Chryseobacterium* sp. (94%)	C	Yellow
168T	*Chryseobacterium /Riemerella* sp. (91%)	B	Yellow
169T	*Pseudomonas* sp. (91%)	B	Yellow
171T	*Chryseobacterium/Haloanella* sp. (95%)	B	Bright white
172T	*Pseudomonas* sp. (91%)	G	White
182T	*Chryseobacterium* sp. (92%)	G	White
201T	*Chryseobacterium* sp. (93%)	F	White

M = marine source; T = terrestrial source

small. Alternatively it is also possible that either bacteria play a relatively minor role in carbohydrate degradation or that some of the possible degraders are viable but not culturable in the laboratory. A vast majority of bacteria in ocean environments are not culturable (Oliver 1993). Efforts are now underway in our laboratory to isolate fungi with such degradative abilities.

Earlier work indicates that microbes with novel enzyme activities can be isolated from marine environments (Greene and Freer 1986). Freer and coworkers have isolated a unique biglucanase from a marine symbiotic bacterium *Teredinobacter turnerae* (Freer pers. comm.). It is not clear whether such enzymes are indeed capable of degrading celluloses in the absence of any other enzyme but this represents one of the enzymes that one is likely to encounter. Additionally, poly (3-hydroxybutyrate-co-3-hydrovalerate) (PHBV) as well as starch degraders have been isolated from tropical environments (Leathers *et al*. 2000). In our experiments most of the carbohydrate degraders isolated could grow and demonstrated degradability both in acidic as well as neutral pH. This is surprising in the case of marine bacteria since their environ-

ment is usually at pH 7.8 or higher (Altman and Dittmar 1966). While this indicated the versatility of these bacteria, whether these degradative abilities are due to one set of pH stable enzymes or due to different enzyme sets is not known at this time. Attempts to assay extracellular enzymes from these isolates yielded no detectable activity (data not shown). This observation, coupled with the fact that the organisms are able to grow in selective media with the respective carbohydrate as a sole source of carbon, is perplexing but not unusual. Similar observations have been made for PHBV depolymerase activities in *Pseudoalteromonas* species (Leathers *et al.* 2000) and amylase activities from *Vibrio* sp. (Franqui 2001). In the latter case cultures had to be grown in 10 L fermenters and showed barely detectable activities, which were evenly divided between extracellular and intracellular fractions (Franqui 2001). It is thus possible that the enzymes associated with carbohydrate degradation in these organisms may be cell associated rather than extracellular. The gram-negative cell is well adapted for life in the low nutrient aqueous environment of the sea because its degradative enzymes are cell wall associated (Costerton *et al*. 1974). Cellulosomes, cell organelles with cellulose binding and degrading activities have also been identified in *Clostridium thermocellum* (Lamed and Bayer 1988).

Currently attempts are being made to culture larger volumes (10 liter fermentations) of the bacterial strains for the isolation and characterization of these carbohydrases.

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RESUMEN

Se aislaron varias bacterias de las islas de Puerto Rico, Vieques y Trinidad por su actividad de degradar carbohidratos. Estas bacterias terrestres y marinas fueron colectadas de suelos para cultivo de piña, bosque lluvioso tropical, costas y pantanos de manglar. Utilizando sustratos de carbohidratos con enlaces cromogénicos (AZCL® Megazyme International Ltd., Ireland) se seleccionaron aquellos organismos que poseían actividad. La composición del medio para el cultivo efectivo de los organismos marinos fue estandarizada. Organismos Gram negativo fueron identificados por análisis de secuencia de la amplificación parcial por PCR del gen 16S de la subunidad pequeña del ARNr. Los resultados indican que la mayoría de los organismos marinos estudiados pertenecen al género *Vibrio* y *Pseudoalteromonas* y que en los ambientes terrestres predominó *Chryseobacterium*. Estos experimentos revelaron que los ambientes subtropicales son potencialmente buenas fuentes de microorganismos con novedosas actividades de carbohidrasas.

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