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SHORT COMUNICATION

CHARACTERIZATION OF RUBBER DEGRADING ISOLATES

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ABSTRACT

Sixteen soil samples were screened for the presence of rubber degrading strains. Twenty-five strains displaying clear zone formation on latex agar plates were purified. Identification revealed that twenty three of these isolates were *Streptomyces* species, one *Pseudonocardia* species and one *Methylibium* species. The addition of carbon sources with the exception of tween 80 enhanced extracellular rubber biodegradation. Scanning electron microscopy revealed that the isolates were able to colonize and penetrate vulcanized glove rubber.

Keywords: latex, rubber, biodegradation, *Streptomyces* sp.

INTRODUCTION

Latex is an important commercial polymer with approximately 10 million tons harvested annually to produce over 40 thousand products **(Jendrossek *et al.*, 1997; Mooibroek and Cornish, 2000)**. Latex consists of rubber particles (*cis*-1,4 polyisoprene) and a small percentage of non-rubber constituents (protein, carbohydrates and salts)

suspended in an aqueous serum **(Othmer, 1997)**. For commercial purposes this polymer normally undergoes a process of vulcanization, altering its molecular structure through the cross-linking of isoprene chains. The abundant use and consequent extensive waste generation of this material has enhanced interest in the area of rubber degradation for the purpose of bioremediation. Actinomycetales have dominated the literature with regard to *cis*-1,4 polyisoprene degradation, with all rubber degrading isolates except three identified as members of this order. *Streptomyces, Nocardia* and *Gordonia* are the most prominent genera. Contrastingly, rubber degradation among Gram negatives is rare. Thus far, just three Gram negative strains namely, *Xanthomonas* sp. 35Y, *Pseudomonas citronellolis,* and *Actinobacter calcoaceticus* possess this ability **(Bode *et al.*, 2000; Bode *et al.*, 2001).** However, it has been suggested that Gram negative isoprene degraders are infrequently isolated due to the absence of growth factors in the media used for screening purposes **(Jendrossek *et al.*, 1997)**. The clear zone isolation technique developed by Spence and Niel is used to detect bacteria which secrete extracellular enzymes during *cis*-1,4 isoprene degradation **(Jendrossek *et al.*, 1997)**. This method selects for latex-utilizing strains on the basis of the formation of translucent halos (clear zones) around colonies on opaque latex agar. *Streptomyces*, *Actinoplanes* and *Micromonospora* fall into this category **(Jendrossek *et al.*, 1997)**. The objective of this study was the isolation and characterization of rubber degrading bacteria from soil.

MATERIAL AND METHODS

Polymers

Two rubber polymers were used: liquid LATZ (low ammonia latex containing tetramethylthiuram disulfide and zinc oxide) provided by the Rubber Research Institute of Malaysia and ExamTex Plus powdered latex gloves (Ansell, Malaysia).

Latex and rubber glove preparation

LATZ was prepared by adding the liquid latex to an equal volume of 0.05% Tween 80. The suspension was centrifuged (10 000 rpm; 10 min.) and the upper cream layer extracted. This was used to make latex agar plates. Latex glove pieces were sterilized in 70% methanol, rinsed in sterile water and added to enrichment cultures.

Culturing of mixed soil samples

One gram of soil was added to 25 ml of X1 stock III solution (g/L): K_2HPO_4. $3H_2O$ 9.17; KH_2PO_4 2.68; $MgSO_4$ 0.1; NH_4Cl 1 in an Erlenmeyer flask. Latex glove pieces, 2-5cm in diameter were added to each flask. This was placed on a rotating shaker (30 rpm) and incubated at 30^oC. Sub-culturing (transferal of the latex glove piece to fresh media) was done after the first month. Thereafter, stock III solution was routinely added to the cultures.

Culturing and isolation of latex utilizing strains

Mixed cultures were streaked onto latex agar (g/L) NH_4Cl 1, agar 15, X10 stock III solution 100 ml, LATZ 1000 µl. Colonies which exhibited zones of clearing on opaque latex agar plates were purified by streaking until a pure strain was obtained. For scanning electron microscopy and Schiff's reagent staining, latex glove pieces were sterilized as described previously and added to minimal media liquid inoculated with the bacterial cultures. These were incubated on a 30 rpm shaker at 30^oC for a month.

Identification based on 16s rDNA sequencing

The first 500 bp of the 16S rDNA gene was amplified using MicroSeq® 500 primers (Applied Biosystems, Warrington, UK). The following reagents were added into 0.2 cm thick walled PCR tubes: 5 µl x10 Taq buffer, 1.5 µl 50mM $MgCl_2$, 1 µl 10mM dNTP, 1 µl DMSO, 10 µl sterilized water, 2 µl forward primer, 2 µl reverse primer, 2 µl DNA template (100ng/µl) and 0.5 µl Taq polymerase (5U/µl) (MBI Fermentas, Hanover, UK) giving a total reaction volume of 25 µl. The conditions were as follows: denaturation at 95^oC for 30 s, annealing at 60^oC for 30 s and extension at 72^oC for 45 s set at 30 cycles, using a BioRad MJ Mini TM Gradient Thermal Cycler PCR machine. For sequencing a Spectromedix LCC sequencer was used in conjunction with an ABI BigDye Terminator v3.1 sequencing kit. Microbial identification was done by aligning sequences to those in the PubMed public database.

Scanning electron microscopy (SEM)

Colonized latex glove pieces were removed from liquid minimal media cultures following 3 months of incubation for use in SEM. To observe colonization the glove pieces were fixed directly. All samples were fixed in 3% gluteraldehyde and left overnight. Once the fixative was drawn off using a Pasteur pipette the treated samples were dehydrated in a graded ethanol series (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and 100%). Consequently, these were subject to critical point drying, mounted onto aluminum stubs by means of carbon discs and lined with graphite. Additionally, these were sputter coated with a thin layer of gold and palladium and viewed under a scanning electron microscope with an electron acceleration setting of 20kV.

Additional carbon source added to latex agar

Individual carbon sources (1%) which include glucose, succinate, fructose, tween 80, mannitol, sucrose, arabinose, xylose, maltose and inositol were added to latex agar plates and the formation of clear zones monitored.

Substrates used as carbon sources

Carbon substrates (nylon, lignin, iron, copper or cobalt at 0.02 %) were added directly to minimal media plates (g/L) NH_4Cl 1, agar 15, X10 stock III solution 100 ml. Cells were washed in sterile water before being spotted onto relevant plates and analyzed routinely for growth.

RESULTS AND DISCUSSION

Collection of soil samples

Sixteen soil samples collected from regions in America, Europe and Africa were screened for the presence of rubber degrading strains (Fig 1). Soil samples were collected from various environments and from these twenty five isolates were purified using an enrichment technique.

Figure 1 Illustration of regions where soil samples were collected .

Isolation and identification of extracellular rubber degrading strains

Twenty five strains displaying clear zone formation on latex agar plates were purified. All isolates were broadly characterized on the basis of rubber degradation. Below are images of two potent extracellular rubber degraders (Fig 2).

Figure 2 Purified strains streaked onto latex agar plates, isolates (right) Chiba and (left) HZ

Noticeably, strains Chiba and HZ were strong extracellular degraders, with the zones of clearing extending far beyond the periphery of the cells. Isolate HZ was the only Gram negative isolated.

PCR (polymerase chain reaction)

Further taxonomic identification was carried out by means of PCR amplification of the first 500 bp of the 16S rDNA region using commercial primers. Ten of the isolates were chosen for species identification. The sequencing of the partial 16S rDNA PCR products and alignment to those in the Entrez PubMed Blast Database revealed the following percentage identity as shown in Table 1. The other fifteen strains based on morphological characteristics were tentatively identified as members of the genus *Streptomyces*.

Table 1 Identification of bacteria using partial 16S rDNA sequences

Strain	Genus and species identification	Percentage similarity to identified species
Gam	*Streptomyces aureus* strain 3184	100
Reu	*Streptomyces pseudogriseolus*	99
Chiba	*Streptomyces flavogriseus*	99
Berlin	*Streptomyces prasinus*	99
Yeo	*Streptomyces griseus* subsp. *griseus*	99
SY6	*Amycolatopsis orientalis*	98
HZ	*Methylibium fulvum*	98
HZWS	*Streptomyces prasinus*	99
BA1	*Streptomyces tendae*	100
Est	*Pseudonocardia* sp.	98

Effect of carbon sources on clear-zone formation

Supplementary carbon compounds were added to latex agar media and its effect on the formation of clear-zones examined (Table 2). As seen in the table below, with the exception of Tween 80, other carbon sources in general did not affect enzyme activity. The only exceptions were *A. orientalis* SY6 and *Streptomyces* sp. Hunt's activity which were repressed in the presence of most carbon sources.

Table 2 Consequence of additional carbon compounds on rubber biodegradation

	Carbon source (1% w/v)								
	Sucrose	**Mannitol**	**Arabinose**	**Xylose**	**Glucose**	**Maltose**	**Inositol**	**Fructose**	**Tween 80**
Strains									
BA1	+	+	+	-	+	+	+	+	-
Gam	+	+	+	+	+	+	+	+	-
Reu	+	+	+	+	+	+	+	+	-
Berlin	+	+	+	+	+	+	+	-	-
Hak	+	+	+	+	+	+	+	+	-
Cal	+	+	+	+	+	+	+	+	-
Pasa	+	+	+	+	+	+	+	+	-
Bot1	+	+	+	+	+	+	-	+	-
FHome	+	+	+	+	+	+	+	+	-
WitsP	+	+	+	+	+	+	+	+	-
SY3	+	+	+	+	+	+	+	+	-
SY5	+	+	+	+	+	+	+	+	-
SY6	-	-	-	-	-	-	-	-	-
HY	+	+	+	+	+	+	+	+	-
WITS	+	-	-	-	-	+	+	+	-
Bedd	+	+	+	+	+	+	+	+	-
H2	+	+	+	+	+	+	+	+	-
H3	+	-	+	+	+	-	+	+	-
BotY	+	-	-	+	+	+	+	+	-
Chiba	+	+	+	+	+	+	+	+	-
Yeo	+	-	+	+	+	-	+	+	-

Scanning electron microscopy (SEM)

Noticeably, after nine weeks all strains had formed dense biofilms covering the entire glove surface and no free cells were detected in the liquid media. SEM imaging was used to investigate colonization and surface modification.

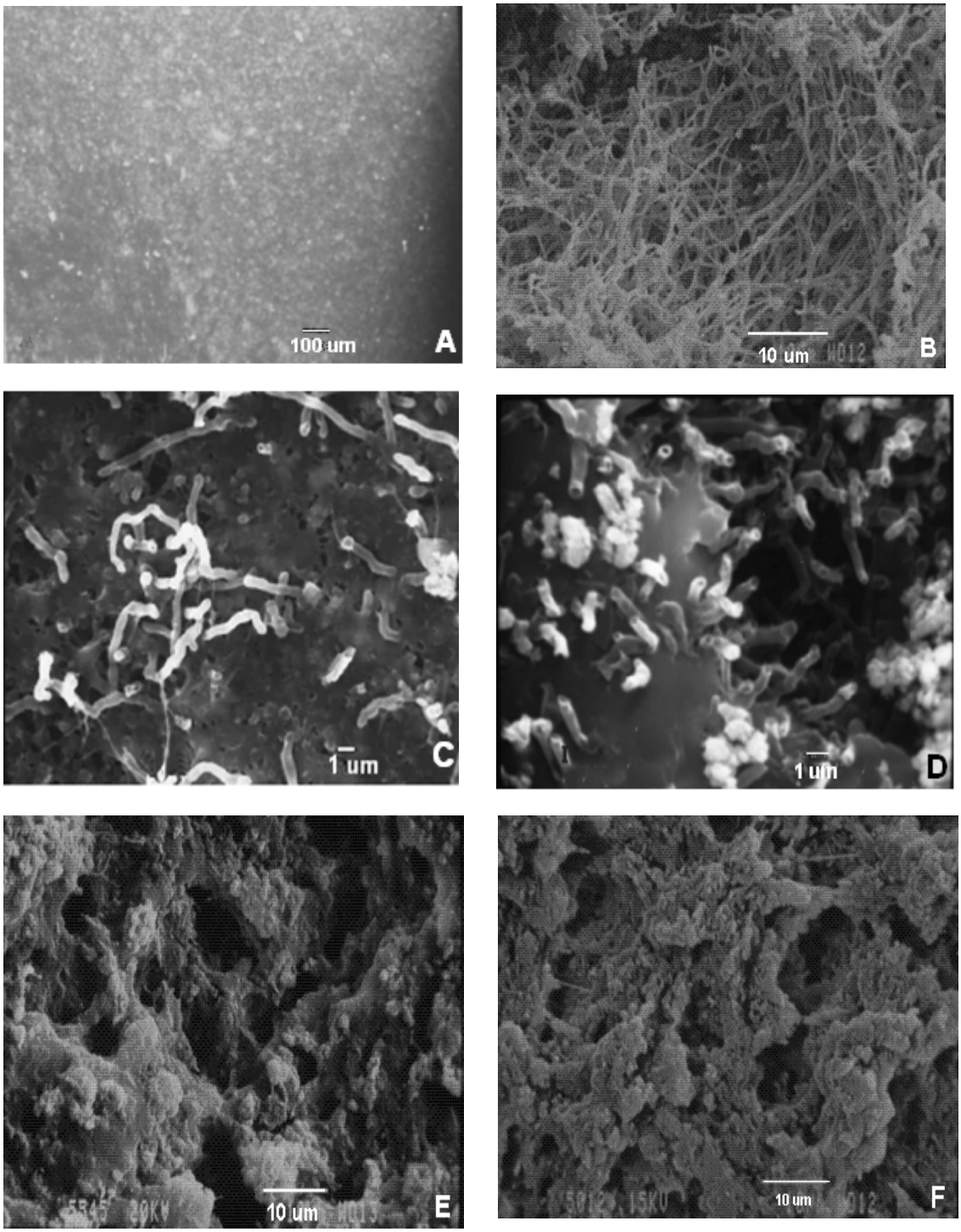

Figure 3 A-F: SEM of natural latex glove pieces following microbial inoculation after nine weeks. (A) uninoculated control, (B) biofilm formation by *S. prasinus* Berlin, (C) rubber surface modification by *Pseudonocardia* sp. Est, (D) *S. tendae* BA1 penetration into the rubber substrate (E) Colonization of glove piece by *S. griseus* Yeo and (F) *A. orientalis* SY6

The uninoculated control glove piece remained uncolonized (Fig. 3A). The *S. tendae* biofilm abundantly covered the rubber and merged into the polymer (Fig. 3D). Likewise, the *Pseudonocardia* sp. biofilm spread across the substrate and was embedded in the material as visualized by the uneven surface (Fig. 3C). *S. prasinus* strain Berlin formed a loose mycelial mat which covered the substrate surface (Fig. 3B). *A. orientalis* SY6 and *S. griseus* Yeo formed similar loose biofilms over the surface of the rubber piece (Fig. 3E and Fig. 3F).

Staining with Schiff's reagent

Strains were grown in liquid media containing rubber glove pieces. Colonized rubber pieces were stained with Schiff's reagent following nine weeks of incubation. The purple coloration indicative of the presence of aldehyde groups due to microbial decomposition of the polymer extended across the whole surface for all twenty five strains tested (data not shown)

DISCUSSION

Rubber is a fairly recalcitrant hydrocarbon compound **(Roy *et al.*, 2006)**. The mixture of chemicals added to enhance its properties and cross-linking contributes further to its resistant nature. Thus, not only must microbes be able to degrade vulcanized bonds, but also resist a plethora of additives. Yet bacteria have evolved pathways to catabolize this compound. Rubber degrading bacteria are abundant and have been isolated from diverse environments in many regions across the world. These include both soil and water samples collected in parts of Asia, Europe and Africa **(Jendrossek *et al.*, 1997)**.

In this study latex agar plates were used to strictly select for extracellular enzyme releasing rubber decomposers, identified by the formation of translucent halos on an opaque background. Resultantly, twenty five strains were isolated and four chosen for detailed characterization.

Partial sequencing of the 16S rDNA gene of one strain showed a 99.8% sequence similarity to both species *S. tendae* and *S. tritolerans*. Subsequently, research was conducted on both strains to identify any distinguishing phenotypic features. A detailed study conducted by Syed and coworkers (2007) using phenetic properties and genetic techniques showed that despite a 99.6% similarity of the complete 16S rDNA gene (three nucleotide

differences) between *S. tendae* and *S. tritolerans* there remained clear disparity between each strain. Three clearly discernible and easily testable traits found in *S. tritolerans* and not shared by *S. tendae* were tolerance towards salinity, alkalinity and temperature. Notably, it possessed the ability to grow at a temperature of 45^{O}C, tolerate a pH of 10 and sodium chloride concentration of 7%. When the streptomycete strain BA1 was tested it displayed no tolerance to any of these factors, supporting its classification as *S. tendae*.

The 16S rDNA sequence linked strain Est to the family *Pseudonocardiacea.* The isolate to which it was matched was however not characterized further to the species level. Thus, Bergey's manual was used to classify the strain **(Holt, 1982; Holt *et al.*, 2000)**. Phenotypic characteristics were matched to the species *Pseudonocardia*. The zigzag shaped hypha is a characteristic feature of this species. In this work just one Gram negative rubber degrader was isolated; as observed in many studies, isolation is rare **(Jendrossek *et al.*, 1997)**. The strain identified as *Methylibium fulvum* HZ displayed a strong extracellular activity, much like the only well characterized Gram negative rubber degrader, *Xanthamonas* sp. 35Y **(Tsuchii and Takeda, 1990)**.

The rubber degrading potential of *Pseudonocardia* sp. has not previously been reported, although members of this genus have been tested. Of 37 *Pseudonocardia* strains analyzed by Jendrossek and coworkers (1997) none displayed a polyisoprene degradative ability. It was not surprising that the majority of the rubber degrading isolates from this study were identified as members of the species *Streptomyces*. Previous studies have shown that this species tends to be the most commonly isolated with regard to rubber decomposition. This was observed in a study conducted by Jendrossek and co-workers (1997) whereby the screening of 1220 bacteria on latex agar led to the isolation of 46 rubber degrading isolates of which 31 were Streptomycetes.

The strain *S. tendae* has been studied previously and is of interest as it produces nikkomycin, a fungicide and insecticide **(Evans *et al.*, 1995)**. It also secretes streptofactin, a biosurfactant which induces aerial mycelia **(Richter *et al.*, 1998).** Members of the species *Pseudonocardia* have been linked with varied features such as fatty acid catabolism, biodegradation of tetrahydrofuran and cellulose production **(Chen *et al.*, 2005; Kohlweyer *et al.*, 2000; Malfait *et al.*, 1984)**.

Actinomycetes exhibit tremendous metabolic diversity, with an ability to degrade a vast array of both natural and xenobiotic compounds and have been implicated in the

degradation of polycyclic aromatic hydrocarbons, pesticides and recalcitrant plastics **(Harada *et al.*, 2006; Lee *et al.*, 1991; Miller *et al.*, 2004)**. However, none of these strains displayed an ability to efficiently utilize any of the diverse carbon elements as sole carbon sources. These isolates were incapable of utilizing alcohols, lignin or nylon as a carbon source and were strongly inhibited by heavy metals (data not shown).

To test whether an alteration in the nutritional composition of the latex media would affect clear zone formation, one extra carbon source was added. Glucose and succinate were tested since these were reported previously as repressing rubber degrading enzymes in most strains. Investigations concerning the regulation of enzyme activity were conducted by Jendrossek and coworkers (1997) on latex degraders. The authors stated that from 47 *Streptomyces* sp. examined, 35 were inhibited by succinate and 45 inhibited by glucose. Also, fructose and mannitol were the only carbon sources which had no effect on enzyme expression. Results recorded here did not show a similar pattern. Apparently, none of the strains enzyme production was affected by the addition of glucose, succinate or fructose. However, Tween 80 repressed clear zone formation. These results were peculiar since many bacteria exhibit catabolite repression, as it is more energy efficient to metabolize simpler carbon sources than a complex hydrocarbon. Yet supplementation of latex agar with glucose resulted in an enhanced clearing zone, suggesting instead that these isolates were utilizing both carbon sources.

Colonization of rubber pieces by strains BA1, Est, Chiba and Yeo was evident due to the intense purple color of Schiff's. As discussed at length by Heisey and Papadatos (1995) the colonization of the hydrocarbon does not definitively constitute an ability to utilize the substrate as an energy source. The presence of non-rubber constituents is enough to sustain the growth of organisms **(Rook, 1955)**. Hence, it is necessary to either demonstrate a weight loss or microscopic modification of the material. Accordingly, SEM was used to monitor colonization and surface modification. All four rubber degrading strains formed dense biofilms, penetrating into the polymer and altering the surface. This is similar to observations made by Heisey and Papadatos (1995), who examined *Streptomyces* sp. modification of the material using SEM. Contrary to what was reported with respect to *Streptomyces* sp. K30, when glucose was added to cultures of strains BA1, Est, Chiba and Yeo containing latex glove pieces, none of the strains colonized the rubber. This demonstrated that minimal nutrient conditions triggered colonization.

Notably, the glove pieces colonized by all four strains retained the same shape and composition (no additional stickiness occurred). Although fully colonized these isolates failed to mineralize the glove rubber. This is in accordance with other studies concerning extracellular rubber degraders such as *Xanthomonas* sp., *Streptomyces coelicolor* 1A, and *Streptomyces* sp. S1G which induced small weight losses of vulcanized rubber by approximately 10 % **(Bode *et al.*, 2001)**. Since the polymer remained intact this suggested as other studies have that these strains are either incapable or inefficient at breaking vulcanized bonds or effected by antimicrobial chemicals **(Linos *et al.*, 2000)**. The inhibition of rubber degrading isolates by antioxidants was well characterized by Berekaa and coworkers (2000) who found the removal of these compounds enhanced both colonization and disintegration of latex gloves. Taking the case of *Pyrococcus furiosus,* it was able to efficiently utilize sulfur thus weakening the vulcanized bonds. Nonetheless, this strain was sensitive to rubber additives, reducing its applicability **(Bredberg *et al.*, 2001)**.

It should be noted that while enzyme-releasing rubber degraders are weak decomposers this does not imply that they are conclusively of no use. It might be possible to employ these bacteria in biotechnological recycling at a later stage. For instance, it is possible to break the vulcanized cross-links using adhesive degraders and use enzyme releasers to further degrade and catabolize the resulting by-products. Alternately, detoxifying bacteria may be used to pretreat the material in preparation for decomposition by these isoprene degrading bacteria.

CONCLUSION

This work was conducted with the purpose of characterizing four rubber degrading isolates. As shown in other studies regarding extracellular degraders, characterization revealed weak rubber biodegraders. Also, this is the first report of a *Methylibium* sp. possessing the ability to degrade latex.

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