IDENTIFICATION AND CHARACTERIZATION OF RUBBER DEGRADING ACTINOBACTERIA

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Abstract. Of a great concern, the huge amount of waste rubber materials can cause environmental problems. Various methods have been proposed to solve this proplem. One of those is the biodegradation of it by microorganisms. Bacteria able to degrade and use natural rubber latex as the sole source of carbon and energy were isolated from different ecosystems. 42 rubber-degrading bacteria were isolated. Out of these isolates, 31 were identified as Streptomyces, 5 as Micromonospora, 3 as Actinoplanes, 2 as Gordona and 1 as Nocardia species. All rubber-degrading isolates were identified as members of Actinobacteria which is a large group of mycelium forming Gram-positive bacteria. Interestingly no Gram-negative bacteria could be isolated.
Keywords: *Streptomyces, Micromonospora, rubber degradation, isolation*

Introduction

Waste rubber is becoming a world wide waste disposal problem [13]. One particular concern is used it because of the huge number of natural rubber product produced and discarded annually and of potential environmental hazard should a NR stock pile catch on fire [14]. Consequently, it is very important and worth trying to develop a microbial process for waste NR disposal [6]. Natural rubber is consisting mainly of cis-1,4-polyisoprene and it is synthesized by more than 2000 plant species belonging mostly to the Euphorbiaceae. NR is still produced in large amounts (~ 10^7 tons / year) from the rubber tree *Hevea brasiliensis*. The cis-1,4-polyisoprene, with an average molecular mass about 10^6 Da, is the main constituent (> 90% of dry weight) of NR. NR is relatively resistant to microbial decomposition compared with many other natural polymers. Since the study by Sohngen and Fol, many reports have been published on the biodegradation of natural rubber by microorganisms [15, 10, 19, 18, 12 and 20]. NR contains a minimum of 90% rubber hydrocarbon together with small amounts of proteins, resins, fatty acids, sugars, and minerals [23]. Organic impurities in the rubber can support microbial growth [2 and 22]. Although many studies have been issued during the last decades on microbial degradation of rubber, only little is known about the occurrence of NR-degrading bacteria. Actinomycetes were almost the only organisms able to considerably decompose NR and use the hydrocarbon as a sole source of carbon and energy [4].

The present study was initiated to isolate and characterize a number of NR-degrading bacteria from various ecosystems in Egypt. It also suggests that rubber-degrading bacteria might be useful for the disposal of discarded rubber products. Identification and development of rubber metabolizing microorganisms potentially could provide a biotechnological solution to this problem.

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Materials and Methods

Sampling sites

This study has concentrated on isolation of NR degrading actinobacteria from some localites in Egypt (*Fig 1*). The sampling sites were collected from various ecosystems (soils and fresh water from the River Nile as well as its bottom sediments).

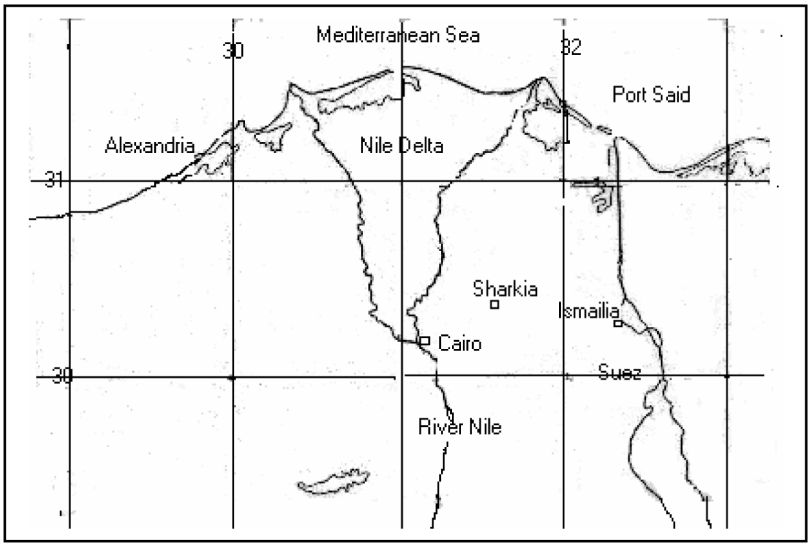

Figure 1. *Location map*

Natural rubber source

Latex of *Hevea brasiliensis* was obtained from Weber and Schaer (Hamburg, Germany).

Isolation and Identification of NR-degrading bacteria

Microorganisms were isolated on mineral salts medium {8.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.5 g $(NH_4)_2$ SO_4, 0.2 g Mg SO_4x$7H_2O$, 0.1g NaCl, 0.1g Ca $(NO_3)2$, 20 mg $CaCl_2$x$2H_2O$, 20 mg of $FeSO_4$x$7H_2O$, 0.5 mg Na_2MoO_4xH_2O and 0.5 mg $MnSO_4$ / L of deionised water} containing 25 to 100 mg of yeast extract and 20 g of agar / L that had been surface coated with a thin film (20-30 mg) of natural rubber. A hexane solution was also applied at the same medium and it was allowed to evaporate under a microbiological hood. Samples from different localities of Egypt from various ecosystems (soils, fresh water and their bottom sediments) were collected. The samples were serially diluted with sterile mineral medium and spread onto mineral plates coated with rubber and hexane and incubated for several weeks at 28 °C. The obsarved colonies were streaked onto the same rubber-coated plates until pure cultures were obtained.

Taxonomic characterisation of isolates

The isolates were characterised according to [1, 21 and 17] schemes based on their macro- and micromorphological properties. Adequate phenotypical tests set and chemotaxonomical investigations were used for the identification of strains including colony and micromorphological characteristics, pigment production tests, whole cell sugar pattern, cell wall chemotype, lecithinase, lipolysis, proteolysis, hydrolysis of pectin, chitin,

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hippurate, casein, esculin, gelatine, degradation of xanthine, elastein, arbutin, utilization of sucrose, m-inositol, mannitol, L-rhamnose, raffinose, d-ribose, salicin, glucose, arabinose, fructose, xylose, galactose, nitrate reduction and H_2S production. The SPSS for Window release 6.0 statistical software has been used for clustering of the isolates, similarity calculations were based on simple matching coefficient (S_{SM}; [16]). The results obtained were further evaluated using the above mentioned different systematic and determinative bacteriological manuals.

Qualitative assay method

Sugars (glucose, fructose, arabinose, sucrose, xylose, inositol, mannitol, rhamnose and raffinose) were sterilised by membrane filtration. 5% of each sugar was supplemented to the same mineral medium. Development or absence of the clearing zone formation was recorded.

Results and discussion

Isolation of NR-degrading bacteria

Ten samples from different ecosystems were screened for the presence of NR-degrading bacteria. 42 NR- degrading bacteria were isolated. They were identified as indicated by (i) size of the colonies developed on solid medium with purified NR latex as the sole source of carbon and energy in comparison to control a plate without NR and (ii) the appearance of translucent halos around the colonies. Interestingly all NR-degrading isolates belonged to the Actinobacteria and no Gram negative bacteria were isolated. Such results are in accordance with those reported by [4 and 20]. The first example of a Gram-negative rubber degrading bacterium, a Xanthomonas species, was reported by [19]. Linos et al. reported a new Gram- negative bacterium species namely *Pseudomonas aeruginosa* for NR-degradation [11]. However, our results cannot exclude NR-degradation capabilities encoded by Gram-negative bacteria in general. Potential Gram-negative NR-degraders might just require additional growth factors or degrade NR by co-metabolism.

Characterisation of NR-degrading bacteria

The isolates were divided into two main groups based on their colony morphology: the polysporic “streptomycetes” and the monosporic or non sporulating “other actinobacteria”. The members of the first group (31 strains) the isolates produced a yellow to grey coloured aerial mycelium with rectusflexiblis or spiral spore chains. The streptomycete isolates were identified as *Str. griseus*, *Str. rochei*, *Str. coelicolor* and *Str. Halstedii* according to the identification schemes of [21 and 17] and taking into consideration the scheme of Bergey’s Manual. Only 5 isolates were Streptomyces sp. due to low similarity indices (*Table 1*).

Members of the “other actinobacteria” group could be divided into 4 genera. The analysis of the first clusters (5 strains) showed morphological features such as well-developed, branched, septate mycelium with a diameter about 0.5 µm, and non-motile single spores, which were characteristic to the genus Micromonospora according to [8]. The members of this cluster showed an orange (young cells) to black–coloured (old cells) substrate mycelium phenotype, and no aerial mycelium was formed. After subse-

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Table 1. Phenotypic and metabolic properties of NR-degrading actinobacteri.

Strain No.	Source	Species	NR	Hexadecane	Glucose	Fructose	Arabinose	Sucrose	Xylose
NR1	S	Str. griseus	+	-	+R	+	-	-	±
NR2	S	Str. griseus	+	-	+R	+	-	-	±
NR3	S	Str. griseus	+	-	+R	+	-	-	±
NR4	S	Str. griseus	+	-	+R	+	-	-	±
NR5	S	Str. griseus	+	-	+R	+	-	-	±
NR6	S	Str. griseus	+	-	+R	+	-	-	±
NR7	FW	Str. griseus	+	-	+R	+	-	-	±
NR8	FW	Str. griseus	+	-	+R	+	-	-	±
NR9	FW	Str. griseus	+	-	+R	+	-	-	±
NR10	FW	Str. griseus	+	-	+R	+	-	-	±
NR11	FWD	Str. griseus	+	-	+R	+	-	±	±
NR12	S	Str. rochei	+	-	+R	+	+	-	+
NR13	S	Str. rochei	+	-	+R	+	+	-	+
NR14	S	Str. rochei	+	-	+R	+	+	-	+
NR15	FW	Str. rochei	+	-	+R	+	+	-	+
NR16	FW	Str. rochei	+	-	+R	+	+	±	+
NR17	FWD	Str. rochei	+	-	+R	+	+	-	+
NR18	FWD	Str. rochei	+	-	+R	+	+	-	±
NR19	S	Str. coelicolor	+	-	+R	+	+	-	-
NR20	S	Str. coelicolor	+	-	+R	+	+	-	-
NR21	S	Str. coelicolor	+	-	+R	+	+	-	-
NR22	S	Str. coelicolor	+	-	+R	+	+	±	-
NR23	FW	Str. coelicolor	+	-	+R	+	+	-	-
NR24	FW	Str. halstedii	+	-	+R	+	+	±	-
NR25	FWD	Str. halstedii	+	-	+R	+	+	-	-
NR26	FWD	Str. halstedii	+	-	+R	+	+	-	±
NR27	S	Streptomyces sp.	+	-	+R	+	+	-	+
NR28	S	Streptomyces sp.	+	-	+R	+	+	-	+
NR29	S	Streptomyces sp.	+	-	+R	+	+	-	+
NR30	FW	Streptomyces sp.	+	-	+R	+	+	-	+
NR31	FWD	Streptomyces sp.	+	-	+R	+	+	-	+
NR32	S	Micromonospora aurantiaca	+	-	±R	-	+	-	-
NR33	FW	Micromonospora aurantiaca	+	-	±R	±	+	±	±
NR34	FWD	Micromonospora aurantiaca	+	-	+R	±	+	±	±
NR35	FWD	Micromonospora aurantiaca	+	-	+R	±	+	±	±
NR36	FWD	Micromonospora aurantiaca	+	-	+R	±	+	±	±
NR37	FW	Actinoplanes italicus	+	-	±R	-	+	±	±
NR38	FWD	Actinoplanes italicus	+	-	+R	+	+	+	±
NR39	FWD	Actinoplanes italicus	+	-	+R	+	+	+	±
NR40	S	Gordona sp.	+	-	+R	±	+	+	-
NR41	FW	Gordona sp.	+	-	+R	±	+	+	-
NR42	S	Nocardia sp.	+	-	+R	±	+	-	-

S: Soil, FW: Fresh water, FWD: Fresh water sediment +: good growth/halo formation
±: poor growth/halo formation -: same growth as on mineral medium without carbon source/no halo
RF: spore chain rectusflexibilies SP: spiral spore chain R: repression of natural rubber degrading activity

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Cont.

Strain No.	Source	Species	Inositol	Mannitol	Rhamnose	Raffinose	Aerial mycelium	Substrate mycelium	Spore chain
NR1	S	Str. griseus	-	+	-	-	yellow	light brown	RF
NR2	S	Str. griseus	-	+	-	-	yellow	light brown	RF
NR3	S	Str. griseus	-	+	-	-	yellow	light brown	RF
NR4	S	Str. griseus	-	+	-	-	yellow	light brown	RF
NR5	S	Str. griseus	-	+	-	-	yellow	light brown	RF
NR6	S	Str. griseus	-	+	-	-	yellow	light brown	RF
NR7	FW	Str. griseus	-	+	-	-	yellow	light brown	RF
NR8	FW	Str. griseus	-	+	-	-	yellow	light brown	RF
NR9	FW	Str. griseus	-	+	-	-	yellow	light brown	RF
NR10	FW	Str. griseus	-	+	-	-	grey	light brown	RF
NR11	FWD	Str. griseus	-	+	-	-	grey	light brown	RF
NR12	S	Str. rochei	+	+	+	-	grey	dark brown	SP
NR13	S	Str. rochei	+	+	+	-	grey	dark brown	SP
NR14	S	Str. rochei	+	+	+	-	grey	dark brown	SP
NR15	FW	Str. rochei	+	+	+	-	grey	dark brown	SP
NR16	FW	Str. rochei	+	+	+	-	grey	dark brown	SP
NR17	FWD	Str. rochei	+	+	+	-	grey	dark brown	SP
NR18	FWD	Str. rochei	+	+	-	-	grey	dark brown	SP
NR19	S	Str. coelicolor	-	+	-	-	yellow	dark brown	RF
NR20	S	Str. coelicolor	-	+	-	-	yellow	dark brown	RF
NR21	S	Str. coelicolor	-	+	-	-	yellow	dark brown	RF
NR22	S	Str. coelicolor	-	+	-	-	yellow	dark brown	RF
NR23	FW	Str. coelicolor	-	+	-	-	yellow	dark brown	RF
NR24	FW	Str. halstedii	+	+	-	-	grey	dark brown	RF
NR25	FWD	Str. halstedii	-	+	+	-	grey	dark brown	RF
NR26	FWD	Str. halstedii	-	+	+	-	grey	dark brown	SP
NR27	S	Streptomyces sp.	+	+	+	-	grey	light brown	SP
NR28	S	Streptomyces sp.	+	+	+	-	grey	light brown	SP
NR29	S	Streptomyces sp.	+	+	+	-	grey	light brown	SP
NR30	FW	Streptomyces sp.	+	+	+	-	grey	dark brown	RF
NR31	FWD	Streptomyces sp.	+	-	+	-	grey	dark brown	RF
NR32	S	Micromonospora aurantiaca	-	-	-	+	-	orange	mono-sporic
NR33	FW	Micromonospora aurantiaca	-	-	-	+	-	orange	mono-sporic
NR34	FWD	Micromonospora aurantiaca	-	-	-	+	-	orange-black	mono-sporic
NR35	FWD	Micromonospora aurantiaca	-	-	-	+	-	orange-black	mono-sporic
NR36	FWD	Micromonospora aurantiaca	-	-	-	+	-	orange-black	mono-sporic
NR37	FW	Actinoplanes italicus	-	-	-	+	-	orange	mono-sporic
NR38	FWD	Actinoplanes italicus	-	-	-	+	-	orange	mono-sporic
NR39	FWD	Actinoplanes italicus	-	-	-	+	-	orange	mono-sporic
NR40	S	Gordona sp.	-	-	-	+	-	orange to red	mono-sporic
NR41	FW	Gordona sp.	-	-	-	+	-	orange to red	mono-sporic
NR42	S	Nocardia sp.	-	-	-	+	-	orange	mono-sporic

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quent analysis of this cluster, it could be identified as *M. aurantiaca*. Koch et al. described this bacterium as a new species [9].

The second cluster was identified as Actinoplanes (3 isolates). Colonies had orange colour and produced pink to cherry coloured pigments diffusing into the medium. According to the Bergey's Manual of Systematic Bacteriology, this cluster is *Actinoplanes italicus*.

The third cluster was identified as Gordona (2 isolates). These isolates had rod shaped or coccoid cells. The genus Gordona is assigned to the suprageneric but phylogenetically coherent group of mycolic acid containing Actinobacteria [7]. These two isolates need more molecular analysis to reach the species level.

The last cluster (one isolate) had mycelia that eventually fragmented into rod shaped or coccoid cells. These characteristics class this isolate into the genus Nocardia. The present strain seems to be a newtype strain of the genus Nocardia and it needs more investigation.

This study demonstrates that certain bacteria can use the hydrocarbon of NR as a sole source of carbon and energy. These microorganisms may play an ecological role in the environment by mineralising NR latexes. Although no attempt was made to isolate the Actinobacteria selectively, all of the isolated rubber metabolising microorganisms were identified as Streptomyces, Micromonospora, Gordona and Nocardia. It suggests that degradation of NR is the privilege of mycelium forming microorganisms. Our results are consistent with those indicate that rubber degrading species of these genera are widely distributed in soils, water and fresh and marine sediments [5, 23 and 3]. Some of these isolates may have enough degrading potential that enables biotechnological use particularly in rubber products.

Regulation of NR degradation

The ability of NR-degrading bacteria to use low molecular mass monomers and high molecular mass polymer as carbon sources was tested (*Table 1*). When clearing zone formation was studied on NR plates containing one additional soluble carbon source, evidence for inhibition of NR degrading enzyme expression was obtained. Carbon sources that allow good growth e.g. glucose repressed the NR-degrading enzyme in most strains (*Table 1*). The extent of inhibition varied with strains and substrates. The biochemical mechanism of NR degradation has not been investigated. Since NR is a high molecular mass compound, which is too large to be taken by bacteria, as a first step the polymer has to be cleaved extracellularly. The extracellular nature of such enzyme system was shown by the appearance of translucent halos on latex containing solid media.

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