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Isolation of Microorganisms Able To Metabolize Purified Natural Rubber

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Bacteria able to grow on purified natural rubber in the absence of other organic carbon sources were isolated from soil. Ten isolates reduced the weight of vulcanized rubber from latex gloves by >10% in 6 weeks. Scanning electron microscopy clearly revealed the ability of the microorganisms to colonize, penetrate, and dramatically alter the physical structure of the rubber. The rubber-metabolizing bacteria were identified on the basis of fatty acid profiles and cell wall characteristics. Seven isolates were strains of *Streptomyces*, two were strains of *Amycolatopsis*, and one was a strain of *Nocardia*.

Natural rubber, consisting mainly of *cis*-1,4-polyisoprene, is relatively resistant to microbial decomposition by comparison with many other natural polymers. Nonetheless, a number of microorganisms have been reported to deteriorate natural rubber and to grow in association with it (1, 9, 11, 17, 27, 28). Tsuchii et al. reported a *Xanthomonas* strain that excreted a rubber-degrading enzyme (26) and a *Nocardia* strain able to use natural rubber as its sole carbon source (25).

The mere presence of microorganisms on or in rubber, however, does not constitute proof of an ability to use the rubber hydrocarbon as a source of carbon and energy. Natural rubber contains a minimum of 90% rubber hydrocarbon, plus small amounts of proteins, resins, fatty acids, sugars, and minerals (28). Organic impurities in the rubber could support microbial growth even if the rubber hydrocarbon itself were not metabolized (2, 27). It is also possible that microorganisms using impurities as their carbon and energy sources could deteriorate the rubber as a result of cometabolism without actually using the rubber hydrocarbon as a source of energy (2). Therefore, unequivocal demonstration of microbial use of rubber as a sole source of carbon and energy requires the use of rubber that is highly purified. Rigorous criteria for verifying the metabolism of the purified rubber will include demonstration of a significant weight loss of the rubber and microscopically observable alteration of its physical structure.

The present study was initiated to verify whether microorganisms can metabolize the natural rubber hydrocarbon. Its objectives were to determine whether microorganisms are able to use highly purified natural rubber as a sole source of carbon and energy, to microscopically characterize the degradation of the rubber, and to identify the microorganisms involved. This work provides insight into the ability of microorganisms to damage commercial supplies of natural rubber (5, 18) and rubber products (2, 9, 11, 28). It also suggests that rubber-degrading microorganisms might be useful for the disposal of discarded rubber products. Rubber tires, which currently contain 35 to 40% natural rubber (8), pose a serious environmental problem because methods for their recycling or disposal are inadequate. Identification and development of rubber-metabolizing microorganisms potentially could provide a biotechnological solution to this problem.

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MATERIALS AND METHODS

Microorganisms were isolated on mineral salts medium [8.0 g of K_2HPO_4, 1.0 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 0.1 g of $Ca(NO_3)_2$, 20 mg of $CaCl_2 \cdot 2H_2O$, 20 mg of $FeSO_4 \cdot 7H_2O$, 0.5 mg of $Na_2MoO_4 \cdot H_2O$, and 0.5 mg of $MnSO_4$ per liter of deionized water] containing 25 to 100 mg of yeast extract and 20 g of agar per liter that had been surface coated with a thin film (20 to 30 mg) of pale crepe rubber (Buffalo Weaving and Belting Co., Buffalo, N.Y.). The rubber was applied as a hexane solution, and the hexane was allowed to evaporate under a microbiological hood. Serially diluted soil samples were spread onto the rubber surface and incubated several weeks at 28°C. Colonies that developed were transferred to other rubber-coated plates until pure cultures were obtained.

The pure cultures were tested for the ability to grow on purified rubber in the absence of additional organic nutrients. Glass microscope slides (7.6 by 2.5 cm), bent at a right angle 1 cm from one end, were coated with natural rubber (ca. 20 mg per slide) by dipping them into a hexane solution of rubber. The rubber had previously been purified by extraction in a Soxhlet apparatus with 250 to 300 solvent cycles of 90% (vol/vol) methanol-water followed by 550 to 600 cycles of acetone. Two coated slides were placed into each sterile glass petri dish and allowed to air dry for 2 to 7 days. Before use, the slides and petri dishes had been incinerated at 550°C for 8 h to remove any traces of organic matter. Sufficient sterile mineral salts medium (as previously described but lacking yeast extract or other organic carbon sources) was added to cover the lower half of the slides. The first series of slides was inoculated with pure cultures grown on rubber-coated mineral salts agar. A second series of slides was inoculated with culture medium (2 ml per slide) taken from the first series of slides after 4 weeks of incubation. The slides were incubated at 28°C and periodically observed for growth.

Isolates exhibiting good growth on the rubber-coated slides were tested for the ability to metabolize vulcanized natural rubber. Rubber from latex gloves (Flexam Floor/Exam Latex Gloves, catalog number 8852; Baxter Healthcare Corp.) was cut into 5-by-0.5-cm strips and purified by rinses in 12 500-ml volumes of deionized water followed by three 30-min soaks in 500-ml volumes of methanol, three 30-min soaks in 500-ml volumes of acetone, and three 10-min soaks in 500-ml volumes of dichloromethane. The leached strips were soaked again in 500 ml of methanol, rinsed thrice with deionized water, drained, and dried at 55°C for at least 3 days to remove all traces of solvent. Six rubber strips (ca. 200 mg total) and 25 ml of mineral salts medium (as previously described but lacking yeast extract or other organic carbon sources) were placed into 125-ml flasks that had previously been incinerated for 8 h at 550°C. The flasks were loosely capped with incinerated glass lids, autoclaved, inoculated (with 3 ml of medium and cells from the second series of rubber-coated slides), and incubated at 28°C. Weight losses of the rubber strips and protein production were determined after 6 weeks. Prior to protein measurement, cells attached loosely to the rubber were dislodged into the broth by boiling the cultures for 15 min, and this was followed by sonication for 15 min and vigorous shaking (300 reciprocations per min) for 10 min. The rubber strips were then removed. Protein was extracted from the cells by adding sufficient NaOH to the culture broth to bring its NaOH concentration to 1 N and then by boiling the broth for 5 min. Results for this protein fraction are under "In

TABLE 1. Weight changes of rubber strips and protein concentrations produced by rubber-metabolizing isolates[a]

Isolate	Wt change of rubber strips (%)[b]	Protein concentration (mg/g of rubber)[c]		
		In culture broth	Of rubber strips	Total
Control	1 ± 1 (a)	0 ± 0	1 ± 0	1 ± 0
S6B	1 ± 0 (a)	2 ± 0	1 ± 0	2 ± 0
S6H	0 ± 2 (a)	2 ± 0	1 ± 0	2 ± 0
S1F	−8 ± 1 (b)	24 ± 9	3 ± 1	26 ± 11
S3G	−9 ± 1 (b and c)	29 ± 2	6 ± 0	35 ± 3
S3D	−11 ± 2 (b, c, and d)	21 ± 7	5 ± 3	27 ± 9
S1A	−11 ± 0 (b, c, and d)	25 ± 3	4 ± 0	29 ± 3
S1D	−12 ± 3 (b, c, and d)	23 ± 4	4 ± 1	27 ± 4
S4C	−12 ± 1 (b, c, d, and e)	26 ± 6	2 ± 1	28 ± 7
S3F	−13 ± 1 (b, c, d, and e)	20 ± 3	12 ± 1	32 ± 2
S4G	−14 ± 2 (c, d, e, and f)	38 ± 2	3 ± 1	40 ± 3
S4E	−16 ± 4 (d, e, and f)	37 ± 4	2 ± 0	39 ± 4
S1G	−16 ± 4 (d, e, and f)	44 ± 9	2 ± 0	46 ± 9
S4F	−16 ± 2 (e and f)	43 ± 3	2 ± 1	45 ± 3
S4D	−18 ± 2 (f)	43 ± 1	3 ± 1	46 ± 2

[a] Isolates were incubated in mineral salts medium for 6 weeks in two or three flasks containing rubber strips.

[b] Data not followed by a common letter differ significantly ($P \leq 0.05$) by Duncan's multiple range test adjusted for unequal replication (12). Values are means ± standard deviations.

[c] Values are means ± standard deviations.

culture broth" in Table 1. Any protein in cells remaining attached to the rubber was extracted by boiling the strips for 5 min in 3 ml of 1 N NaOH. Results for this fraction are under "Of rubber strips" in Table 1. Protein in the extracts was determined by a modified Lowry method (10). The rubber strips were dried at 52°C and weighed after extraction.

Scanning electron microscopy was used to examine the colonization, penetration, and degradation of latex from rubber gloves by isolates S1G, S4D, and S3F. The former two isolates were chosen because they were among those causing the greatest weight loss of rubber strips and the greatest protein production with rubber as the sole carbon source (Table 1). Isolate S3F, which was intermediate in its ability to degrade rubber, was selected because it was chemotaxonomically very different from the other isolates (Table 2; see also Fig. 3). Cultures for electron microscopy were grown in mineral salts medium containing strips of purified rubber from latex gloves as described above. An initial set of culture flasks was inoculated with spores or cells of the isolates grown on YM agar (4). A second set of cultures was inoculated 14 days later with liquid medium (1 ml per flask) from the previous flasks and grown for 45 days at 28°C. Rubber strips from the second set of cultures were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0 to 7.2), postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, and dehydrated with a graded ethanol series. Pieces of the rubber were chilled in liquid nitrogen and cryofractured to expose edges for examination. Samples were critical point dried (Bio-Rad EBS model E3000), sputter coated (BAL-TEC model SCD 050) with gold and palladium, and examined with a scanning electron microscope (JEOL model JSM 5400).

Isolates causing a >10% weight loss of the rubber strips were identified to genus level. Cells were grown for 5 days at 25 to 30°C in shaken flasks of yeast extract-dextrose broth (15) and were separated from the broth, dehydrated in ethanol, and dried at 20 to 40°C. Whole-cell hydrolysates were prepared and analyzed for diaminopimelic acid (DAP), diagnostic sugars, and mycolic acids according to the methods of Kutzner (14). Fatty acid profiles were determined by a capillary gas chromatography method (Microbial Identification, Inc., Newark, Del.) described by Sasser (23). The fatty acid profiles of the rubber-degrading isolates were compared by computer with those in databases of known microorganisms. A dendrogram based on fatty acid content was calculated with an algorithm by cluster analysis techniques.

RESULTS AND DISCUSSION

Fourteen cultures isolated on rubber-coated mineral salts agar grew on glass slides coated with purified natural rubber in mineral salts medium. The ability to grow under these conditions indicates the isolates were able to use the purified rubber as their sole source of carbon and energy. It should be noted, however, that certain actinomycetes (e.g., *Nocardia* [*Amycolata*] *autotrophica* and *Nocardia* [*Amycolata*] *saturnea*) can live chemoautotrophically on atmospheric CO_2, or CO_2 and H_2, as well as metabolize organic compounds (16). Therefore, merely demonstrating an ability to grow in the presence of purified rubber without also showing a change in the weight or other physical characteristics of the rubber cannot be considered presenting unequivocal evidence that the rubber is used as a sole source of carbon and energy.

Ten isolates reduced the weight of vulcanized rubber from latex gloves by >10% within 6 weeks, and four reduced the weight by >15% (Table 1). The weight of rubber strips in noninoculated control flasks did not change appreciably, indicating that weight loss in the inoculated flasks was due to biological processes rather than to nonbiological oxidation or alkaline extraction during protein measurement.

Protein production was closely correlated with weight loss of the rubber strips (Table 1). For the 10 isolates causing the greatest weight reduction, an average of 26% (a range of 22 to 30%) of the lost weight of the rubber was recovered as total protein. These results are nearly identical to those reported (25) for protein production by a *Nocardia* isolate growing on unvulcanized natural rubber (27%), synthetic isoprene rubber (26%), and rubber bands made from vulcanized natural rubber (26%). The yield of protein with rubber as the sole carbon source is somewhat higher than expected for certain other

TABLE 2. Taxonomic characteristics of isolates causing >10% weight reduction of rubber strips

Isolate	Isomer of DAP	Diagnostic sugars[a]	Mycolic acid[b]	Fatty acid pattern[c]						Genus
				Saturated (C_{14} to C_{18})	Unsaturated (C_{14} to C_{18})	Iso (C_{16})	Iso (C_{15} or C_{17})	Anteiso (C_{15} or C_{17})	10-Methyl (C_{17}/C_{18})	
S1A[d]	*meso*	A, G	−	++	p	++	+	+	p/−	*Amycolatopsis*
S1D[d]	*meso*	A, G	−	+	+	++	+	+	p/−	*Amycolatopsis*
S1G	L	—	NA	++	p	+	++	+++	−/−	*Streptomyces*
S3D	L	—	NA	+	p	+	+	++++	−/−	*Streptomyces*
S3F	*meso*	A, G	+	+	p	p	++++	p	−/−	*Nocardia*
S4C	L	—	NA	++	p	+	+	++++	−/−	*Streptomyces*
S4D	L	—	NA	+	p	++	+	+++	−/−	*Streptomyces*
S4E	L	—	NA	+	p	+	+	+++	−/−	*Streptomyces*
S4F	L	—	NA	++	p	+	+	+++	−/−	*Streptomyces*
S4G	L	—	NA	++	p	+	+	+++	−/−	*Streptomyces*

[a] A, arabinose; G, galactose; —, diagnostic sugars absent.

[b] −, mycolic acid absent; +, mycolic acid present; NA, not applicable.

[c] −, not present; p, 1 to 9%; +, 10 to 19%; ++, 20 to 29%; +++, 30 to 39%; ++++, 40 to 49% of total fatty acid methylesters.

[d] 2-OH-Iso and anteiso C_{15} to C_{17} fatty acid methyl esters are also present (S1A, 2.6%; S1D, 6.4%).

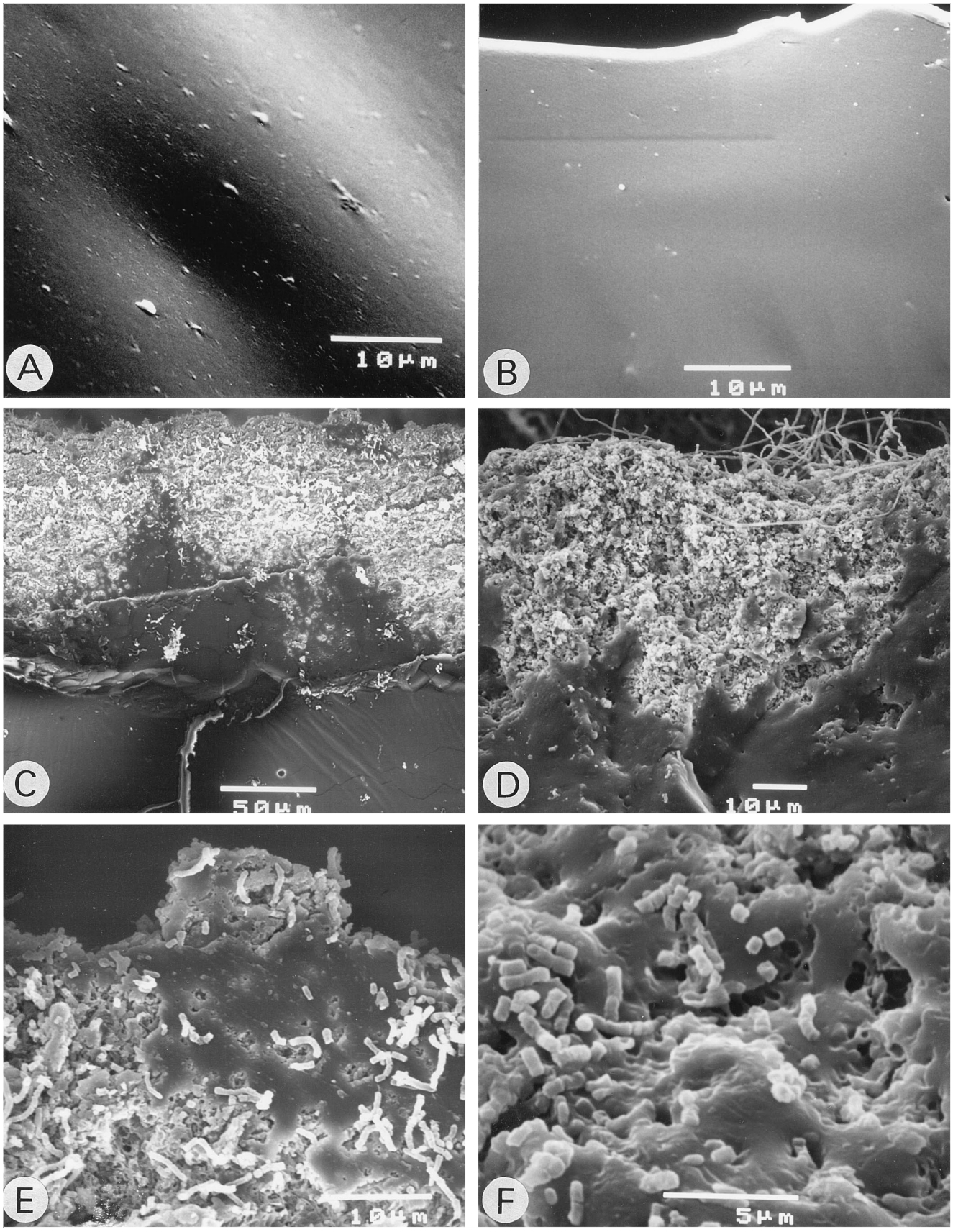

FIG. 1. Scanning electron micrographs of rubber from latex gloves. Micrographs A and B show the surface and fractured edge, respectively, of uninoculated rubber (control). Micrographs C, D, and E (fractured edges) demonstrate the penetration of isolate S1G into the rubber; micrograph F shows the severe deterioration of the rubber surface colonized by isolate S1G.

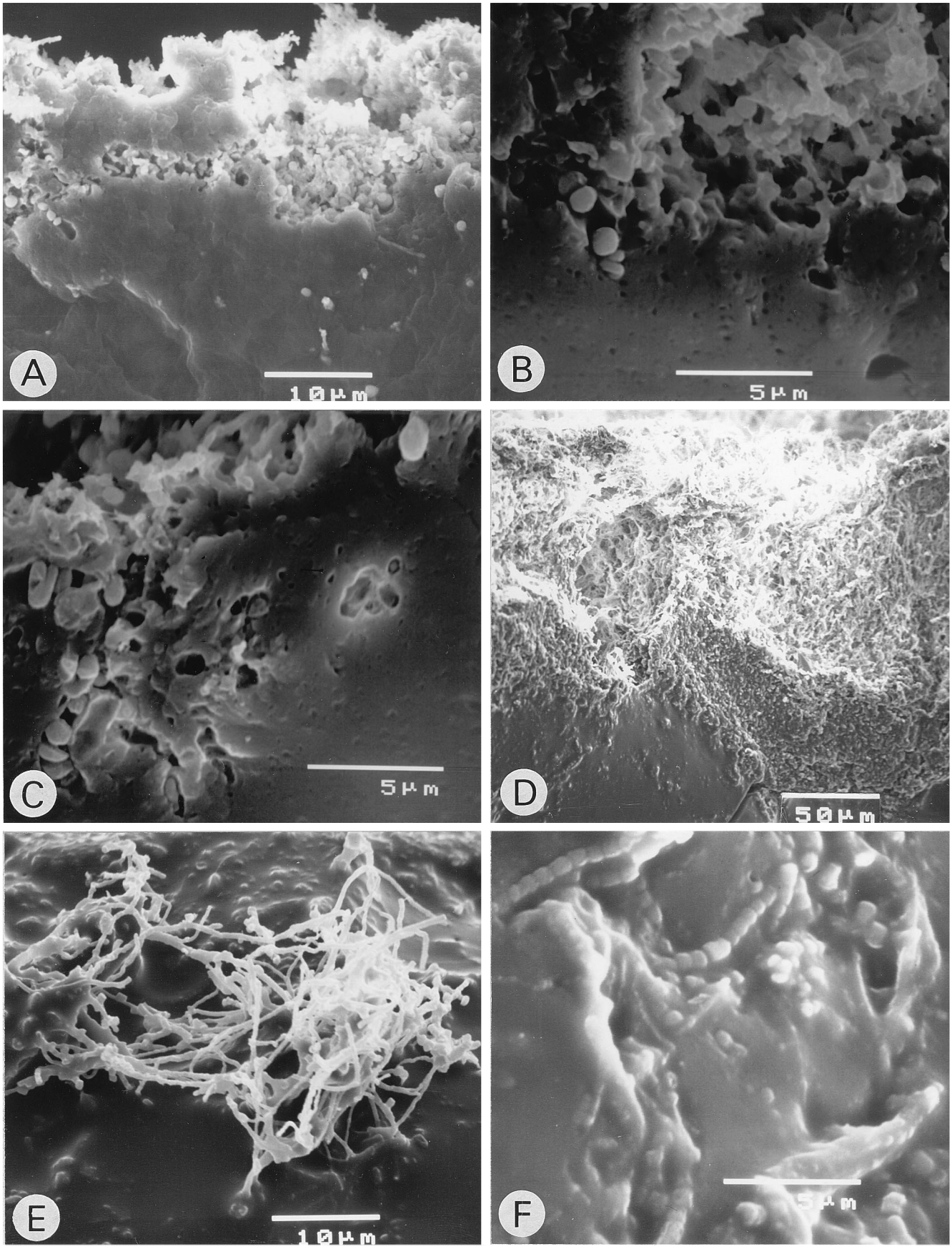

FIG. 2. Scanning electron micrographs of rubber strips from latex gloves. Micrograph A shows a fractured edge, with isolate S3F colonizing the surface (top of photo) and penetrating into the rubber; micrographs B and C show fractured edges, with isolate S3F penetrating into the rubber. Micrograph D shows extensive colonization of the rubber surface (upper half of photo) by isolate S4D, with penetration of the organism into the fractured face of the rubber strip, micrograph E shows isolate S4D and the characteristic pebbling of the rubber surface it caused, and micrograph F shows isolate S4D growing embedded in the rubber matrix.

carbon sources. Aerobic chemoheterotrophs using sugars as a sole carbon source typically convert 20 to 50% of the carbon from the sugar into cellular carbon (24). *Streptococcus faecalis* and *Klebsiella aerogenes* growing aerobically on glucose produced 0.32 and 0.39 g of biomass per g of glucose consumed, respectively (19). Since the protein content of a bacterium is commonly about 50% of the cell dry weight (22), a typical yield of protein from bacteria metabolizing sugars would be 15 to 25% of the weight of sugar metabolized. Although the growth yield from rubber is somewhat higher than that expected for sugars, this result is not surprising, since the rubber hydrocarbon contains more energy per unit weight than do carbohydrates.

A minuscule amount of protein (0.1% of rubber weight) was measured in the uninoculated control. This suggests protein was not completely removed from the rubber strips during purification. The amount remaining, however, was negligible compared with the amount of protein produced by most isolates. These results provide strong evidence that the isolates used the rubber hydrocarbon as their source of carbon and energy in protein synthesis.

Scanning electron microscopy unequivocally demonstrated the ability of the three isolates examined (S1G, S3F, and S4D) to degrade rubber from latex gloves (Fig. 1 and 2). The isolates not only heavily colonized the rubber surface but also extensively penetrated into the rubber during the 45 days of incubation. Severe alteration and deterioration were evident on the surface and within the rubber compared with the condition of the uninoculated (control) rubber (Fig. 1A and B), which remained unaltered during the incubation. Deterioration was characterized by a roughening of the rubber surface (Fig. 1F and 2A and E), development of a granular appearance on fractured edges (Fig. 1C and D and 2D), and an increase in porosity by what appeared to be enzymatic digestion (Fig. 1E and F and 2A to C). Isolates S1G and S4D penetrated into the rubber more deeply than isolate S3F, reaching a depth of 125 μm or more (Fig. 1C and 2D). Isolates S1G (Fig. 1D to F) and S4D (Fig. 2D) produced filamentous growth composed of short rod-shaped cells. Although isolate S3F also exhibited some filamentous growth, individual coccoid cells tended to be more common (Fig. 2A to C).

The ten isolates causing a >10% weight loss of the rubber strips were identified to genus level. All produced gram-positive, filamentous growth and grew on oatmeal agar, starch agar, and YMG agar (11), indicating that they were actinomycetes. Isolates S1G, S3D, S4C, S4D, S4E, S4F, and S4G produced brown or yellow substrate mycelia and white to light gray aerial mycelia and exhibited a powdery gray spore mass characteristic of *Streptomyces* spp. Spore production was not observed for S1A, S1D, and S3F. On YMG agar, S1A and S1D produced light brown substrate mycelia and a medium amount of white aerial mycelia, whereas S3F produced orange-pink substrate mycelia and copious white aerial mycelia.

Isolates S1G, S3D, S4C, S4D, S4E, S4F, and S4G contained L-DAP and lacked diagnostic sugars (Table 2), indicating a type I cell wall and type C whole-cell sugar pattern (15) characteristic of the streptomycetes group (6). They also exhibited fatty acid patterns (Table 2) similar to those of *Streptomyces* spp. (6, 13). A comparison of their fatty acid profiles with those in the Actinl database (20) indicated that they were strains of the genus *Streptomyces*. The dendrogram based on fatty acid content suggested that three species groups of the genus *Streptomyces* were present (Fig. 3).

Isolates S1A and S1D contained *meso*-DAP, with arabinose and galactose as diagnostic sugars (Table 2), indicating a type IV cell wall and type A sugar pattern (15) characteristic of

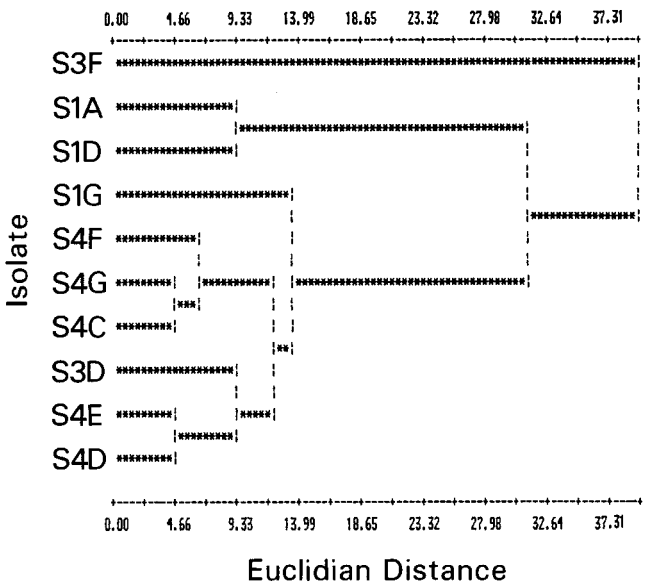

FIG. 3. Dendrogram showing the relationships of rubber-degrading isolates. The dendogram was calculated on the basis of the whole-cell fatty acid contents of the isolates.

nocardioforms (6). They also eventually fragmented into rod-shaped or coccoid cells typical of nocardioforms. Both isolates lacked mycolic acids and had similar fatty acid patterns rich in iso- and anteiso-branched acids (Table 2). Both cultures had been isolated from the same soil sample, and the results indicate that they are probably the same species (Fig. 3). A search of the Actinl and Aerobe databases (20, 21) indicated that the fatty acid profiles of S1A and S1D were most similar to those of *Amycolatopsis* spp. This genus consists of species formerly included in the genus *Nocardia* but which lack mycolic acids and have major amounts of branched-chain fatty acids (3, 16).

Isolate S3F was similar to S1A and S1D in having a type IV cell wall, a type A sugar pattern (Table 2), and mycelia that eventually fragmented into rod-shaped or coccoid cells. Unlike S1A and S1D, it contained mycolic acid. These characteristics place S3F in the genus *Nocardia*. Its fatty acid profile (Table 2), however, was atypical for most *Nocardia* species in that it had very large amounts of branched-chain fatty acids, comparatively small amounts of nonbranched fatty acids, and no detectable 10-methyl (tuberculostearic) fatty acids (7). The dendrogram verified the difference of S3F from the other isolates (Fig. 3). A search of the Actinl and Aerobe databases (20, 21) showed no similar entries, even at the generic level, suggesting that S3F is an uncommon strain of the genus *Nocardia.*

This study demonstrates that certain soil bacteria can use the hydrocarbon of natural rubber as a sole source of carbon and energy. It also shows the ability of the bacteria to cause major degradative changes in the rubber structure. These microorganisms may play an ecological role in the soil by mineralizing latexes produced by certain plants. Although no attempt was made to selectively isolate actinomycetes, all of the rubber-metabolizing microorganisms identified were actinomycetes in the genera *Streptomyces*, *Amycolatopsis*, and *Nocardia*. Our results are consistent with those of other investigations, which indicate that rubber-degrading species of these genera are widely distributed in soil, water, and sewage (2, 9, 11, 17, 25, 27, 28). Some of these isolates may have the potential for biotechnological uses in cases in which the degradation of natural

rubber would be advantageous, such as in the disposal of discarded rubber products. It must be noted, however, that an ability to degrade natural rubber does not necessarily indicate a capability to metabolize synthetic rubber polymers (25).

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