

## Sequential Growth of Bacteria on Crude Oil

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By modification of the enrichment culture procedure three bacterial strains capable of degrading crude oil in sea water were isolated in pure culture, UP-2, UP-3, and UP-4. Strain UP-2 appears to be highly specialized for growth on crude oil in sea water since it showed strong preference for oil or oil degradation products as substrates for growth, converted 66% of the oil into a form no longer extractable by organic solvents, quantitatively degraded the paraffinic fraction (gas chromatographic analysis), emulsified the oil during exponential growth, and produced  $1.6 \times 10^8$  cells per mg of oil. After exhaustive growth of UP-2 on crude oil the residual oil supported the growth of UP-3 and UP-4, but not a previously isolated oil-degrading bacterium, RAG-1. Strains UP-2, UP-3, and UP-4 grew on RAG-1-degraded oil (specifically depleted of *n*-alkanes). The growth of UP-3 and UP-4 on UP-2 and RAG-1-degraded oil resulted in the production of new paraffinic compounds as revealed by gas chromatography. When the four strains were grown either together in a mixed culture or sequentially, there was over 75% oil conversion. By plating on selective media, growth of the individual strains was measured kinetically in the reconstituted mixed culture, revealing competition for common growth substances (UP-2 and RAG-1), enhanced die-off (UP-2), and stabilization (UP-4) during the stationary phase.

There has been a large number of reports (e.g., 2, 5, 13, 14, 17) describing the properties of pure and mixed bacterial cultures capable of degrading and dispersing crude oil in sea water. From these studies, at least three generalizations have emerged. First, in all cases, the ability of the bacteria to significantly degrade oil in sea water is dependent on the addition to the culture of an exogenous source of nitrogen and phosphorus (3); this requirement for added nitrogen and phosphorus compounds most likely accounts for the very slow breakdown of crude oil in the open sea. Second, low-molecular-weight normal alkanes are degraded most rapidly in the laboratory (11) and after oil spills in the open sea (6, 10). Third, mixed cultures carry out a more extensive degradation than pure cultures (7).

In most studies on pure cultures of oil-degrading bacteria, the microorganism was obtained initially by enrichment culture procedures in which maximum specific growth rate or maximum final cell concentration was used as the selection criterion. Thus paraffin oxidizers, which grow rapidly and reach high cell concentrations on crude oil, have been selected and examined in greatest detail. Bacteria which grow more slowly or oxidize minor components

of the crude oil never come to fore in batch enrichments, although the activity of these microorganisms may be of special significance in natural environments. In order to overcome this difficulty, we have used a modification of the enrichment culture technique, in which the fraction of crude oil remaining after exhaustive growth of one bacterium is then used as the carbon source in the subsequent isolation of a second type.

We describe here the isolation of three oil-degrading bacteria by a sequential enrichment culture technique. These bacteria, together with an organism previously isolated in our laboratory, RAG-1 (16), were examined for their ability to grow on crude oil in pure culture or in reconstituted mixtures of pure cultures as well as sequentially. In addition, dispersion and degradation of oil were examined under various growth conditions.

### MATERIALS AND METHODS

**Liquid media.** Unless otherwise stated liquid media contained per liter of filtered sea water:  $K_2HPO_4 \cdot 3H_2O$ , 10 mg; urea, 450 mg; and varying concentrations of Iranian crude oil. The crude oil used in these studies (Rostam) has a specific gravity at 20 C of 0.834. Chromatographic fractionation of the

oil (8) revealed that it contained 61.2% paraffins, 19.9% aromatics and naphthenes, 9.3% polar compounds, and 3.6% asphaltenes. The physical properties of paraffinic oil from this area have been reported (9). In order to check for salinity tolerance, artificial sea water was prepared according to Baumann et al. (4). For special purposes, three additional types of oil were used to prepare liquid media. (i) Heavy heated oil was obtained by treating Ballaym land crude oil from Sinai ( $D = 0.899$ ) in vacuo at 195 to 220 C for 20 h, resulting in a 22.5% decrease in mass and an increase in density to 0.946. (ii) RAG-1-depleted oil was obtained by inoculating strain RAG-1 into the above liquid media containing 0.07% (vol/vol) Rostam crude oil and incubating for 3 days at 30 C. The oil was then extracted with an equal volume of benzene-pentane-ether (3:1:1, vol/vol/vol). The oil remaining after evaporation of the solvent is referred to as RAG-1-depleted oil. (iii) UP-2-depleted oil was prepared exactly as above, except that strain UP-2 was employed in the growth step.

**Solid media.** For examining growth on different carbon sources, SWMA agar was prepared which contained per liter of filtered sea water:  $K_2HPO_4 \cdot 3H_2O$ , 1 mg; urea, 45 mg; and agar, 15 g (Difco). After streaking the different strains onto quadrants of SWMA agar, a carbon source was added to the center, and the plates were incubated at 32 C for 1 week. The carbon sources employed were: hexadecanol, maltose, glycine, L-alanine, L-glutamate, L-tyrosine, L-serine, L-lysine, and L-phenylalanine (Aldrich); D-alanine (Calbiochem); D-galactose and 2-oxo-glutarate (Fluka); naphthalene, glycerol (Frutarom); D-glucose, sodium citrate, sodium benzoate, *n*-pentane, benzene, ethanol (Merck); D-mannose, D-ribose, D-xylose, lactose, L-leucine, L-histidine hydrochloride, L-proline, L-methionine, L-valine, L-aspartate (Sigma); hexadecanoic acid (British Drug House).

For enumerating total viable cell number and distinguishing the different strains, a number of carbon sources were added to SWMA agar. HDYE agar contained 0.1% *n*-hexadecane (Aldrich) and 0.1% yeast extract (Difco); the hexadecane was added to the medium in the form of an emulsion prepared by sonification. UP2DO agar contained 0.07% UP-2-depleted oil; ACYE agar contained 0.5% sodium acetate (Analar) and 0.1% yeast extract; PE agar and HIS agar contained phenylalanine and histidine, respectively; SWN agar was nutrient agar (Difco) prepared with filtered sea water.

**Cell growth.** Unless otherwise stated, incubation was at 29 C with reciprocal shaking. Flasks of 250 ml, 500 ml, and 5 liters were used for 30 ml, 50 ml, and 1 liter of media, respectively. Dilutions for viable cell number were performed with sterile filtered sea water. During exponential growth, strains RAG-1 and UP-2 adhere to oil droplets; thus estimates of viable cell number are minimum values when these strains are grown on crude oil or paraffins. However, during early stationary phase, viable counts were equal to total counts as determined by use of a Petroff-Hauser counting chamber.

**Microorganisms.** Strain RAG-1, an *Arthrobacter* sp., has been described previously (16). UP-2 was obtained by the enrichment culture technique with 50 ml of 0.1% heavy heated oil and 5  $\mu$ g of yeast extract per ml in supplemented sea water as the growth medium. After inoculation with oil sand taken from a local chronically polluted beach (Tel Baruch), the flask was incubated with shaking for 1 week. After two transfers, UP-2 was isolated on nutrient agar from the mixed culture. Strains UP-3 and UP-4 were obtained by the same technique except that UP-2-depleted oil served as the carbon source for the selective culture and UP2DO agar as the medium for isolation. The pure cultures were maintained on HDYE agar by monthly transfers.

**Determination of oil conversion and oil dispersion.** For the purposes of this report, oil conversion is defined as the process by which crude oil in sea water is converted into a form which is no longer extractable by an equal volume of benzene-pentane-diethylether (3:1:1, vol/vol/vol). After extraction, the organic solvent was removed first with a stream of air and then under vacuo at room temperature for 24 h. The difference in weight between the control (no bacteria) and the experimental represents oil conversion. Oil dispersion was measured as previously described (15).

**Gas chromatography.** To chromatographically examine the paraffin fraction, a portion of the residual oil (after estimating oil conversion) was dissolved in a known volume of pentane in order to obtain a concentration suitable for the chosen recorder sensitivity. A Packard-Becker series 417 gas chromatograph, equipped with a dual flame ionization detector and Unicorder U-125 recorder, was used: 10% OV-1 on 80 to 100 mesh Gas Chrom V, 6 feet by 1/8 inch (182.8 by 0.317 cm); column temperature programmed from 50 to 320 C at 7 C per min; injection temperature, 260 C; detector FID temperature, 340 C; carrier gas, nitrogen; chart speed, 10 mm per min; sample size, 3  $\mu$ l.

## RESULTS

**Isolation and general description of oil-degrading bacterial strains UP-2, UP-3, and UP-4.** Enrichment cultures used to isolate crude oil-degrading bacteria yield different strains even after several transfers. The probable reason for this is the heterogeneity of the carbon source. Low-molecular-weight paraffin oxidizers ( $C_{10}$  to  $C_{25}$ ) generally dominate the cultures because of their more rapid growth rate. In order to isolate bacteria which could utilize other fractions of the crude oil, two variations of the enrichment culture technique were successively employed. In the first procedure, heated heavy oil from Sinai, which did not support the growth of strain RAG-1, was used as the carbon source. With this technique (see Materials and Methods for details of enrichment culture procedures), a mixed culture was

obtained which emulsified the heavy oil. The bacterium which dominated the mixed culture was obtained in pure culture by streaking onto SWN agar. This bacterium, referred to as UP-2, grew on and emulsified heavy oil in pure culture. The second procedure employed UP-2-degraded oil as the sole carbon source in the enrichment culture. Utilizing this method, a mixed culture was obtained which grew on but did not emulsify the remaining oil. From this mixed culture, two strains, referred to as UP-3 and UP-4, were isolated in pure culture by streaking onto UP-2DO agar. This selective plate does not support the growth of strains RAG-1 or UP-2. Table 1 compares the growth of four oil-degrading bac-

teria on different carbon sources. RAG-1 grew on 31 of the 35 carbon compounds tested, whereas UP-2 grew only on crude oil, hexadecane, hexadecanol, hexadecanoic acid, acetate, ethanol, sucrose, maltose, or glucose. Strain UP-4 utilized all of the compounds tested except naphthalene. UP-3 grew poorly or not at all on the amino acids tested except for glycine. These data provide the basis for developing selective plates in order to distinguish the four strains in a mixed culture: only UP-4 grows on L-phenylalanine (PE agar); both UP-4 and RAG-1 grow on L-histidine (HIS agar); all grow on acetate (ACYE agar); only UP-2 fails to grow on complex media, such as nutrient agar, in the absence of added NaCl. Alternatively, UP-2 can be distinguished from the other strains by the fact that it alone can grow in NaCl at relatively high concentrations (from 10 to 16%).

UP-2 is a slightly motile gram-negative rod in all media that support its growth; its average dimensions are 2.5 by 0.85  $\mu\text{m}$ . Whereas UP-2 has a narrow range of utilizable carbon sources, it has a wide temperature range (15 to 45 C) and salt tolerance (2 to 16% NaCl). Morphologically, UP-3 and UP-4 are similar to each other. They both are nonmotile gram-negative ovoids, approximately 1.1 by 1.0  $\mu\text{m}$ . As RAG-1, both strains can grow at temperatures up to 42 C either in tap water or in the presence of NaCl at concentrations up to 4 to 6%.

**Kinetics of growth and oil dispersion.** The final cell yield as a function of the concentration of crude oil in the growth medium for each of the newly isolated strains is shown in Fig. 1. The growth of strain UP-2 was directly proportional to oil concentrations up to 0.1% (vol/vol), yielding approximately  $1.6 \times 10^9$  cells per mg of oil. From the dimensions of UP-2 and assuming a density of 1.05 g per ml, one can calculate that 2.8 mg (wet weight) of cells was produced from 1 mg of oil. Growth of strains UP-3 and UP-4 was much poorer than UP-2, yielding only  $1.6 \times 10^8$  and  $3.1 \times 10^7$  cells per mg of oil, respectively, at oil concentrations less than 0.07% (vol/vol). At higher oil concentrations, growth of strain UP-3 was greatly inhibited. Thus, all subsequent growth experiments were performed with oil concentrations not exceeding 0.07% (vol/vol).

The kinetics of growth and oil dispersion were determined for strains UP-2, UP-3, and UP-4, as well as the previously isolated oil-degrading bacterium RAG-1 (Fig. 2-5). The data clearly indicate that strain UP-2 is superior to the other strains with respect to maximum cell yield ( $8 \times 10^8/\text{ml}$ ) and oil emulsification (160 Klett units). Furthermore, UP-2 emulsifies oil during the

TABLE 1. Growth of oil-degrading bacteria on different carbon sources<sup>a</sup>

Carbon source	Oil-degrading strain			
	RAG-1	UP-2	UP-3	UP-4
Crude oil <sup>b</sup>	+	+	+	+
Hexadecane	+	+	+	+
Hexadecanol <sup>b</sup>	+	+	+	+
Hexadecanoic acid <sup>b</sup>	+	+	+	+
Acetate	+	+	+	+
Ethanol	+	+	+	+
D-Glucose	+	±	+	+
D-Galactose	+	-	+	+
D-Mannose	+	-	+	+
D-Ribose	+	-	+	+
D-Xylose	+	-	+	+
Sucrose	+	+	+	+
Lactose	+	-	+	+
Maltose	+	+	+	+
Glycine	+	-	+	+
L-Alanine	+	-	-	+
D-Alanine	+	-	±	+
L-Glutamate	±	-	±	+
L-Leucine	+	-	±	+
L-Histidine-hydrochloride	+	-	-	+
L-Methionine	-	-	±	±
L-Tyrosine	+	-	±	+
L-Serine	+	-	-	+
L-Proline	+	-	±	+
L-Lysine	+	-	-	±
L-Phenylalanine	-	-	-	+
L-Valine	±	-	±	+
L-Aspartate	+	-	±	+
Glycerol	+	-	+	+
Pentane	+	-	+	+
Benzoate	+	-	+	+
Benzene	+	-	+	+
Naphthalene	-	-	-	-
2-Oxoglutarate	-	-	+	±
Citrate	+	-	- <sup>c</sup>	- <sup>c</sup>

<sup>a</sup> Growth was tested on sea water agar media supplemented with the carbon source.

<sup>b</sup> Growth was demonstrated only in shake flasks.

<sup>c</sup> Not checked.

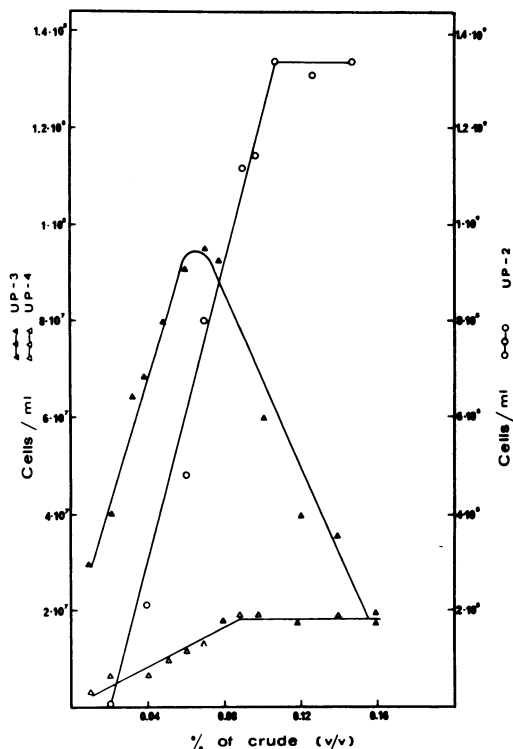


FIG. 1. Growth of oil-degrading bacteria as a function of crude oil concentration. Viable cell number was determined after 72 h for UP-2 and UP-4 and after 80 h for UP-3.

exponential growth phase, whereas RAG-1-induced oil emulsification reaches a maximum only during the stationary phase of growth. Neither UP-3 nor UP-4 brings about significant oil emulsification during its growth on crude oil. The generation times for RAG-1, UP-2, UP-3, and UP-4 on crude oil were 156, 300, 516, and 188 min, respectively.

Microscopic examinations of cultures of UP-2 growing on hexadecane or crude oil revealed the following. Initially, the occasional cell that is seen was attached to large oil droplets; during the exponential growth phase usually one but occasionally two small colonies were seen attached to the surface of most oil droplets; the microscopic colonies varied in size from 4 to 8 cells up to several hundred cells closely packed in a two-dimensional array (Fig. 6). As the culture approached stationary phase, the oil droplets became smaller and cells could be seen both in the medium and on the oil. In stationary phase, most cells were not attached.

**Oil degradation by pure cultures.** Table 2 shows the crude oil conversion by each of the four strains. Once again it can be seen that

UP-2 was the most efficient in converting oil (65.8%) into a form which is no longer extractable by organic solvents. It is interesting to note that although strains RAG-1, UP-3, and UP-4 exhibit only 0.1 to 0.001 the cell yield of UP-2, they still convert 40 to 50% of the oil into a nonextractable form.

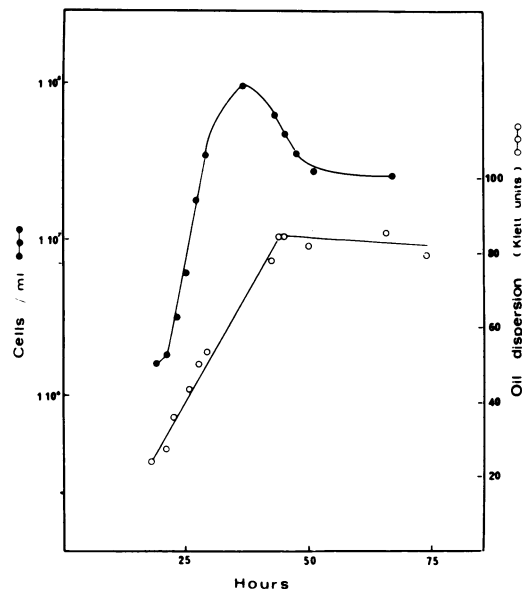


FIG. 2. Kinetics of growth and oil dispersion of RAG-1 on 0.07% (vol/vol) crude oil.

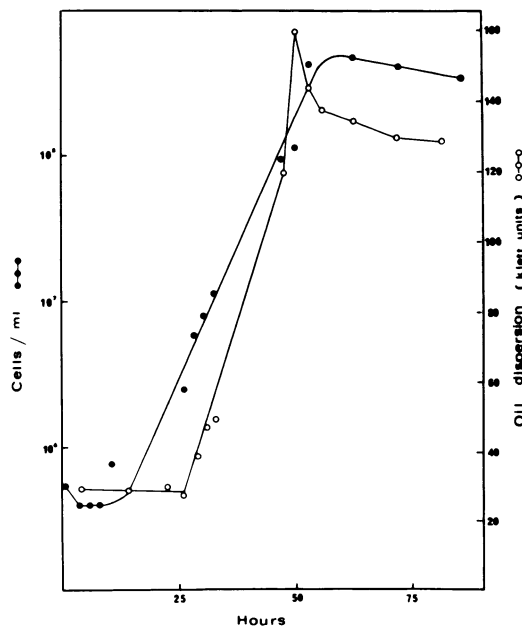


FIG. 3. Kinetics of growth and oil dispersion of UP-2 on 0.07% (vol/vol) crude oil.

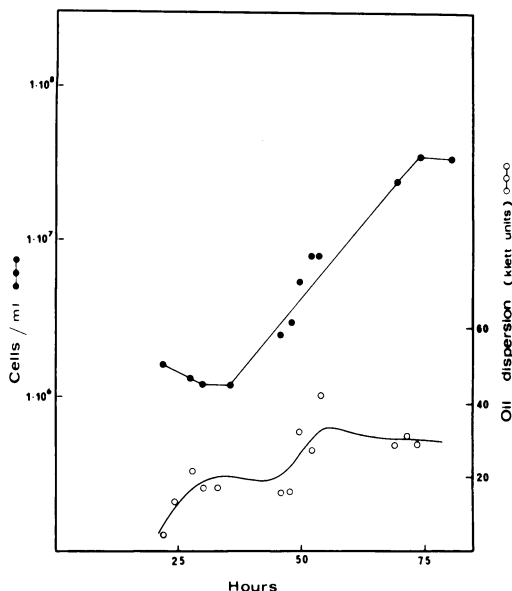


FIG. 4. Kinetics of growth and oil dispersion of UP-3 and 0.07% (vol/vol) crude oil.

The fate of the paraffinic fraction of the crude oil after growth of each of the strains was examined by gas liquid chromatography (Fig. 7). There were no large differences in the chromatographic profiles of the crude oil before (curve A) or after treatment with strains UP-3 (curve D) or UP-4 (curve E). The only significant change brought about by the latter two strains is the appearance of two new peaks, one migrating between tetradecane and pentadecane brought about by UP-3 and the other produced by growth of UP-4, migrating between dodecane and tridecane. RAG-1 degraded most of the alkanes between  $C_{11}$  and  $C_{25}$ , whereas strain UP-2 quantitatively (>95%) degraded the paraffinic fraction as measured by this technique.

**Degradation of crude oil by sequential growth of oil-degrading bacteria.** In order to gain insight into the interaction between the oil-degrading bacteria, the growth of each of the strains was examined on crude oil that had been depleted previously by exhaustive growth of either RAG-1 or UP-2 (Table 3). By comparison with crude oil as a carbon source, RAG-1-depleted oil still supported 67, 71, and 68% the maximum cell yields of UP-2, UP-3, and UP-4, respectively. As a control, RAG-1 produced less than 5% its maximum cell yield on the homologously depleted oil. UP-2-depleted oil was a relatively poor carbon source for growth of RAG-1 (1%) or UP-2 (9%), but a good substrate for subsequent growth of UP-3 (83%) and

UP-4 (159%). Since these experiments were performed under conditions of limiting carbon, it is clear that UP-3 and UP-4 can utilize components of oil not degraded by either RAG-1 or UP-2. This finding is in agreement with the gas chromatographic data (Fig. 7) in which neither UP-3 nor UP-4 brought about any significant changes in the profiles of the paraffinic fraction.

Crude oil was then used as the carbon source for the sequential growth of the four strains (Table 4 and Fig. 8). Step 1: RAG-1 yielded  $8 \times 10^7$  cells per ml and converted 52.3% of the oil into a nonextractable form. The alkanes between  $C_9$  and  $C_{25}$  were degraded extensively in this initial step (Fig. 8, curve A). Step 2: UP-2 converted 42.8% of the RAG-1-degraded oil yielding  $3 \times 10^8$  cells per ml. The chromatographic profile showed the almost complete absence of the paraffinic fraction (Fig. 8, curve B). Step 3: Although UP-3 yielded  $10^8$  cells per ml on the degraded oil, only 3.8% of the remaining oil was converted into a nonextractable form. Interestingly, the chromatographic profile of the remaining oil (Fig. 8, curve C) showed the appearance of two new peaks, eluting between  $C_{20}$  and  $C_{23}$  alkanes. Step 4: In the final step, UP-4 degraded 13.2% of the remaining oil, yielding  $10^7$  cells per ml. Again the chromatographic profile (Fig. 8, curve D) demonstrated the appearance of new peaks. The data indicated continuous degradation of the crude oil, yielding 77.2% oil conversion after the sequen-

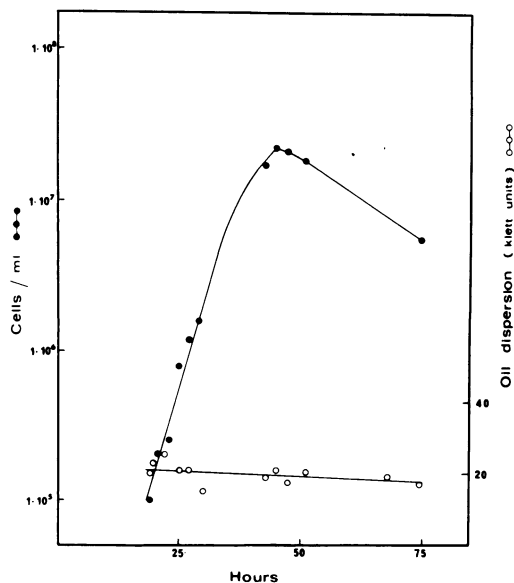


FIG. 5. Kinetics of growth and oil dispersion of UP-4 on 0.07% (vol/vol) crude oil.



FIG. 6. Phase contrast micrograph of strain UP-2 growing in supplemented 0.1% hexadecane sea water medium. Magnification:  $\times 620$  (diameter of oil droplet approximately 150 microns).

tial growth of the four strains. Most of the oil was converted in the first two steps.

**Simultaneous growth of the four strains on crude oil.** By use of selective plates (see Materials and Methods and Table 1), the kinetics of growth of UP-2, UP-3, UP-4, and RAG-1 were monitored after simultaneous incubation of the four strains (Fig. 9). During the first two days, UP-2 dominated the culture, reaching almost  $3 \times 10^8$  cells per ml, and then began to die so that by 4.5 days it was the least abundant viable cell type ( $1.5 \times 10^6$  cells per ml). RAG-1 grew more slowly initially than in pure culture and reached

only about  $3 \times 10^7$  cells per ml. In the later stages, there appeared to be a secondary growth phase, possibly due to breakdown products from UP-2. Populations of UP-3 and UP-4 rose slowly to over  $4 \times 10^7$  cells per ml. Thus, UP-3 and UP-4 reached the same or higher cell concentrations when grown in mixed cultures as in pure culture cultures. Oil conversion was 74% at the end of the experiment.

#### DISCUSSION

By a modification of the enrichment culture technique in which components of crude oil

TABLE 2. Growth and oil conversion by oil-degrading bacteria<sup>a</sup>

Strain	Cells/ml	Extractable oil remaining (g/liter)	% Oil conversion
RAG-1	$1.0 \times 10^8$	0.224	52.8
UP-2	$1.2 \times 10^8$	0.162	65.8
UP-3	$5.0 \times 10^7$	0.263	44.4
UP-4	$2.5 \times 10^7$	0.286	39.7

<sup>a</sup> Five-liter flasks containing 1 liter of supplemented sea water and 0.06% (vol/vol) crude oil were inoculated with approximately  $10^4$  bacteria per ml and incubated at 32 C with reciprocal shaking. After 3 days (4 days for UP-3) viable cell numbers and remaining oil were determined. The data are averages of at least two independent experiments. The control flask (minus bacteria) yielded 0.474 g/liter of extractable oil after 3 days.

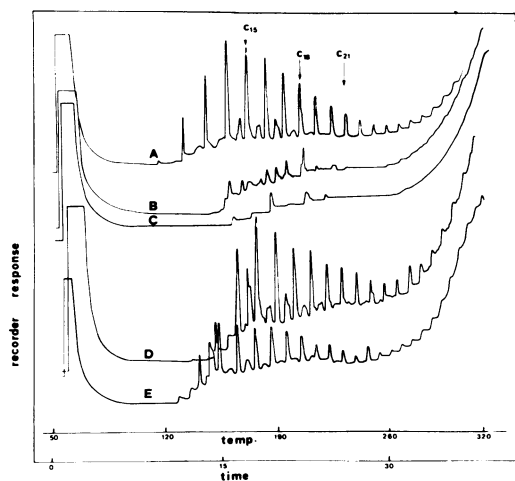


FIG. 7. Gas chromatograms of 0.06% (vol/vol) treated crude oil. (A) Control after shaking for 3 days, without bacteria; (B) after growth of RAG-1 for 3 days; (C) after growth of UP-2 for 3 days; (D) after growth of UP-3 for 4 days; and (E) after growth of UP-4 for 3 days. The samples were extracted and concentrated 20-fold. Sensitivity was  $16 \times 10$ .

were sequentially removed and the remaining oil was used as a substrate for subsequent enrichment procedures, three oil-degrading bacteria were isolated, strains UP-2, UP-3, and UP-4. These bacteria differ in growth characteristics from each other and from a previously isolated oil-degrading *Arthrobacter*, RAG-1. Strain UP-2 appears to be the most specifically adapted to growth on oil in sea water, since it prefers a wide variety of oil components or oil degradation products as substrates for growth to complex media, such as nutrient broth and yeast extract, sugars, amino acids, and other

organic materials tested. Strains UP-3 and UP-4 show a wide range of utilizable substrates, which may explain their ability to multiply on RAG-1- and UP-2-depleted oil.

Comparison of growth of the pure cultures and reconstituted mixtures on crude oil and bacterial "depleted" crude oil has provided information on which components of oil are used by the different strains. Strain UP-2 degraded the largest fraction of the crude oil and yielded the largest number of bacteria per mg of oil. Since UP-2-depleted oil did not support the growth of RAG-1, it is reasonable to assume that UP-2 consumed all of those components which RAG-1 can utilize. The reverse is not true, since UP-2 grows well on RAG-1-depleted oil. These conclusions are supported by the chromatographic data which indicate that RAG-1 consumed the straight-chain paraffins, but did not appreciably degrade the minor broad peaks (probably branch-chain isomers), whereas UP-2 degraded almost quantitatively all the paraffins appearing as peaks on the chromatograms. Clearly, UP-3 and UP-4 degrade components other than the paraffins since they grew well on UP-2-depleted oil. In fact, UP-3 and UP-4 may produce paraffins by some unknown process (Fig. 8, curves C and D) from other fractions of crude oil. However, until these products are chemically characterized it would be premature to speculate on possible mechanisms. Furthermore, it should be emphasized that the chromatographic procedures used in this study only allow for an analysis of a portion of the oil.

Growth kinetics of the mixture of the four strains on crude oil (Fig. 9) was not completely predictable for the growth of the pure cultures and indicate several phenomena of ecological interest. In the early phases of growth, there was a competition between RAG-1 and UP-2, apparently for *n*-alkanes, so that neither strain reached the population densities that were

TABLE 3. Growth of oil-degrading strains on bacterial-depleted oil

Strain	Final cells/ml <sup>a</sup> on oil		
	Crude	RAG-1 degraded	UP-2 degraded
RAG-1	$8 \times 10^7$	$1.7 \times 10^6$	$7 \times 10^6$
UP-2	$4.5 \times 10^6$	$3 \times 10^6$	$4 \times 10^7$
UP-3	$3.5 \times 10^7$	$2.5 \times 10^7$	$3 \times 10^7$
UP-4	$2.2 \times 10^7$	$1.5 \times 10^7$	$3.5 \times 10^7$

<sup>a</sup> Viable cell number was determined at maximum cell yield. In all cases the initial cell concentration was approximately  $10^4$ /ml.

TABLE 4. Sequential conversion of crude oil by bacteria<sup>a</sup>

Strain	Vol (ml)	Extractable oil (mg)		Cells/ml	% Oil conversion	
		Initial	Final		Specific step	Accumulated data
RAG-1	1000	474	226	$8 \times 10^7$	52.3	52.3
UP-2	500	203	116	$3 \times 10^8$	42.8	72.7
UP-3	250	105	101	$1 \times 10^8$	3.8	73.7
UP-4	125	91	79	$1 \times 10^7$	13.2	77.2

<sup>a</sup> RAG-1 was grown on crude oil as described in Table 3. After weighing the extracted oil a portion was suspended in 500 ml of supplemented sea water and inoculated with UP-2. After growing UP-2 on RAG-1-depleted oil for 3 days, the residual oil was extracted and weighed, and a portion was added to 250 ml of supplemented sea water, which was then inoculated with UP-3. After 4 days of growth the residual oil was once again extracted and weighed, and a portion was added to 125 ml of supplemented sea water. The flask was inoculated with UP-4 and incubated for 3 days. The amount of oil remaining after sequential degradation by RAG-1, UP-2, UP-3, and UP-4 was determined.

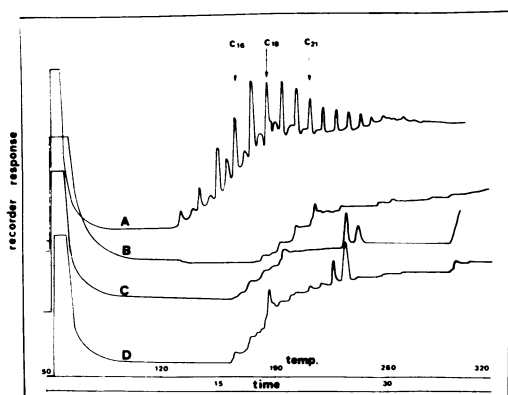


FIG. 8. Gas chromatograms of sequential growth on 0.06% (vol/vol) crude oil. (A) After RAG-1; (B) after UP-2; (C) after UP-3; and (D) after UP-4. After each step, the culture was re-extracted and concentrated 200-fold. Sensitivity was  $32 \times 10$ .

achieved in pure cultures. Then, for unknown reasons, the population of UP-2 declined to less than 1% of its maximum. Such a drop in the population does not occur in pure cultures of UP-2. On the other hand, UP-4 which showed a pronounced death phase in pure culture was somehow stabilized in the mixed culture. Another result that was not predicated was the enhanced growth of UP-3 and UP-3 in the mixed culture. Since these strains can utilize a wide variety of sugars and amino acids, it is possible that they grew on organic matter produced by the death of UP-2 cells. The growth kinetics of the four strains in mixed culture also allow us to better understand the selective pressures involved in the enrichment culture procedure. RAG-1 was the first and easiest strain to isolate because it grows fastest and does not die rapidly under the conditions employed. UP-2 is difficult to isolate by enrich-

ment on crude oil because it is neither the most rapidly multiplying nor most stable. Strains UP-3 and UP-4 only achieve their maximum populations slowly and in mixed cultures. Sequential growth of microorganisms on mixed hydrocarbons has recently been reported (16).

Microbially induced emulsification of oil is poorly understood from both mechanistic and teleological points of view. Microorganisms can utilize crude oil as a substrate for growth with or without concomitant oil emulsification (1, 15). Although there have been some attempts to characterize the emulsifying agents, the preparations have not been purified sufficiently to identify the active components (12, 18). In the present study oil emulsification was obtained during the exponential growth phase with UP-2, stationary phase with RAG-1, and not at all with UP-3 and UP-4. Since strain UP-2 appears to be the most specifically adapted to growth on oil, preliminary microscopic examinations of the culture were performed throughout the growth cycle. The observations have led us to hypothesize that oil emulsification is an integral part of the growth cycle of certain microorganisms. Even at low concentrations, UP-2 becomes tightly attached to fresh oil droplets. The cells then multiply on the surface of the oil, forming small colonies. During exponential growth, the bacteria produce a dispersing agent(s) which breaks up the oil droplets into smaller units, thereby producing new surface area, necessary for the increasing population.

In order to check the reasonability of this hypothesis, one can examine maximum concentration of bacteria as a function of oil droplet radius,  $r$ . Assuming UP-2 lies flat on the oil surface (as observed in phase microscopy) then each cell would occupy  $2.0 \times 10^{-6}$  cm<sup>2</sup> of oil surface. Since the experiments reported here



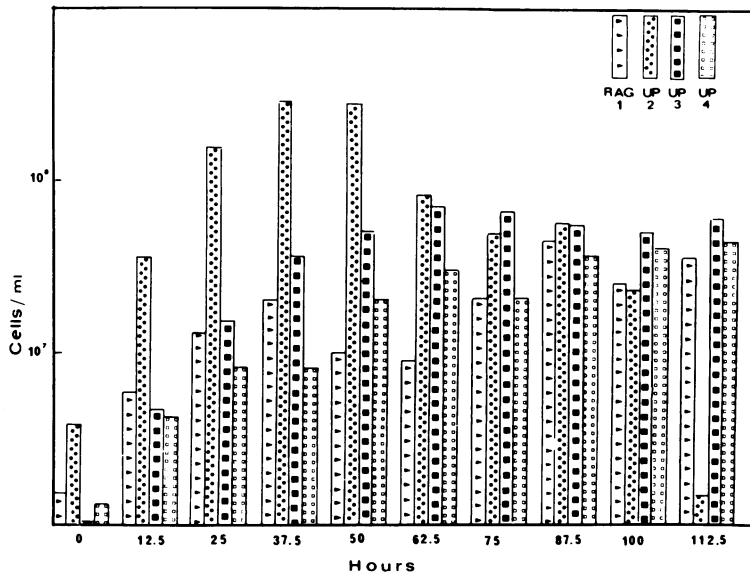


FIG. 9. Kinetics of growth of RAG-1, UP-2, UP-3, and UP-4 in a mixed culture. Supplemented sea water containing 0.06% (vol/vol) crude oil was inoculated simultaneously with each of the above bacteria and incubated at 29 C. At 12.5-h intervals, samples were removed and viable cell concentration for each of the strains was determined.

were performed with 0.07% (vol/vol) oil, the oil surface per milliliter is  $2.1 \times 10^{-3} \text{ cm}^3/\text{r}$ . The maximum number of bacteria per milliliter is then given by  $2.0 \times 10^{-3} \text{ cm}^3/\text{r} \times 2.1 \times 10^{-8}$  or approximately  $10^5 \text{ cm}^3/\text{r}$ . Thus, in order to achieve concentrations of UP-2 greater than  $10^8$  per ml, oil droplet size would have to be reduced to less than  $20 \mu\text{m}$  in diameter. Oil droplets between 5 to  $10 \mu\text{m}$  in diameter are routinely observed during the late stages of UP-2 growth on crude oil. On limiting amounts of crude oil, UP-2 becomes dissociated from the residual nonutilizable oil during stationary growth phase. If our interpretation of these observations are correct, then UP-2 provides another interesting example of how microorganisms are able to alter their microenvironment in order to enhance their growth.

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