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Microbial Degradation of Crude Oil: Factors Affecting the Dispersion in Sea Water by Mixed and Pure Cultures

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By means of the enrichment culture technique, a mixed population of microorganisms was obtained which catalyzed the dispersion of crude oil in supplemented sea water. From this enrichment culture, eight pure cultures were isolated and studied. Only one of the isolates (RAG-1) brought about a significant dispersion of crude oil. RAG-1 has been tentatively characterized as a member of the genus *Arthrobacter*. The other seven isolates gave rise to colonies on supplemented oil agar, but were neither able to disperse oil nor to stimulate the dispersion catalyzed by RAG-1. The dispersion of crude oil by either RAG-1 or the enrichment culture was absolutely dependent on exogenous sources of nitrogen and phosphorous and completely inhibited by 10^{-2} M azide. The increase in cell number of RAG-1 was directly proportional to the concentration of crude oil added to the medium over the range 0.05 to 1.0 mg/ml. Within this linear region, 1.0 mg of crude oil yielded 9×10^7 cells and approximately 65% of the oil was converted into a nonbenzene extractable form. Accompanying the emulsification was a decrease in the *p*H from 7.6 to 5.0. Acidic conditions, however, were neither necessary nor sufficient for oil dispersion. When sea water was supplemented with 0.029 mM K_2HPO_4 and 3.8 mM $(NH_4)_2SO_4$ and inoculated with RAG-1, oil dispersion occurred within 1 day. This dispersion could also be brought about by the supernatant following separation of the cells from the medium. Similarly, the supernatant obtained following growth of RAG-1 on hexadecane was capable of emulsifying crude oil in 60 min.

The possibility of employing microorganisms for the degradation of petroleum and its derivatives in minimizing contamination due to oil leaks and spills has prompted a number of investigators to study the process in the laboratory (2, 3, 5, 6, 8, 9, 12, 14). For example, Miget et al. (8) have recently described the isolation of 50 active oil-degrading cultures (mixed cultures) from sea water and sediments containing crude oil. In the most active preparations, 40 to 55% of the oxidizable crude oil was degraded in enriched sea water in 60 hr. In general, the degradation was accompanied by an emulsification, resulting in a greater oil-water interface. This is significant since there is evidence to indicate that the greater the oil-water interface, the faster the rate of oil decomposition by microorganisms (3, 10, 13).

The present investigation was undertaken in order to study some of the factors which influence the kinetics of microbial dispersion of crude oil in sea water. During the course of this investigation a pure culture was obtained capable of bringing about a rapid dispersion and extensive degradation of oil. The effect of certain factors, such as concentrations of ammonia and phosphate, on the processes catalyzed both by the mixed enrichment culture and the pure culture is reported in this communication.

MATERIALS AND METHODS

Media. Unless otherwise stated, liquid media contained per liter of sea water: K_2HPO_4, 10 mg; $(NH_4)_2SO_4$, 1 g; and varying quantities of crude oil. The sea water was obtained from a local beach (Tel Baruch). Iranian Crude Oil was obtained from the Israel Institute of Petroleum. The physical properties and chemical composition of paraffinic oil from this area have recently been reported (4). The particular oil used in these experiments has a specific

gravity at 15.5 C of 0.842, corresponding to 0.06% asphaltenes, 2.6% carbon residue, and less than 2.0% sulfur (4). Crude oil was sterilized by passage through 0.45-μm membrane filters (Millipore Corp.). In some experiments, the medium was supplemented with yeast extract (Difco) or tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, *p*H 8.0. Nutrient broth consisted of 25 g of no. 2 nutrient broth (Oxoid) and 5 g of yeast extract/liter. The *p*H was adjusted to 7.2. Media were solidified with 1.5% agar (Difco).

Microorganisms. Two types of inocula were utilized in this study. The first group of experiments was performed with a mixed culture obtained by the enrichment culture technique. To 20 ml of unsterilized sea water were added 155 mg of unsterilized crude oil, 100 μg of yeast extract, 0.056 mM K_2HPO_4 and 7.6 mM $(NH_4)_2SO_4$. After about 1 week of incubation at 32 C with shaking, the oil became evenly dispersed throughout the liquid. One milliliter of this culture was then transferred to sterile supplemented sea water containing filtered oil. After one transfer, the oil became dispersed in only 2 to 4 days. Such mixed cultures were maintained by serial transfers to fresh media at 3-day intervals.

The second group of experiments was performed with pure cultures obtained by streaking the enrichment culture onto either nutrient agar or oil agar. Eight different colony types were isolated and found to grow both on nutrient- and oil-containing media. The isolates were maintained by periodic transfer on nutrient agar. In order to obtain a standard inoculum, isolates were grown for 24 hr in nutrient broth, harvested, washed three times, and suspended in saline.

Determination of oil dispersion. To estimate the extent of oil dispersion, culture flasks were shaken vigorously, and 5-ml samples were transferred immediately to 150 by 14 mm test tubes. After allowing the mixture to stand for 2 min, 2 ml was carefully removed from the middle of the liquid column and transferred to micro-Klett tubes for measurement of turbidity in a Klett-Summerson colorimeter fitted with a green filter. Sterile medium served as a blank. Controls in which microbial activity was inhibited by azide yielded readings of less than 5 Klett units (KU). Where oil dispersion was evident (e.g., Fig. 1), turbidities between 100 and 1,000 KU were generally recorded. Turbidity due to bacteria was only about 10 KU per 10^8 microorganisms per ml of either mixed populations or pure cultures.

Determination of oil conversion. For the purposes of this paper, oil conversion is defined as the process by which crude oil is converted to a form which is no longer extractable by benzene. Oil conversion was determined by extracting 300 ml of incubation mixture with 30 ml of benzene; the aqueous phase was extracted a second time with 30 ml of benzene, and the combined benzene fractions were filtered through Whatman no. 1 filter paper in order to clarify the extract. The benzene was evaporated at 37 C to constant weight in a tared large petri dish.

Cell growth. Viable cell numbers were determined by spreading 0.1 ml of an appropriate dilution onto nutrient agar. Colony counts were performed after incubation at 32 C for 4 days. Within experimental error, the viable count was equal to the total cell count as determined by use of a Petroff-Hauser counting chamber.

RESULTS

Dispersion of crude oil by mixed cultures. By utilizing the enrichment culture technique (see Materials and Methods), a mixed culture of microorganisms was obtained which grew on crude oil as the sole source of exogenously added carbon and energy. After 2 to 4 days, the fraction of oil which was not degraded became visibly dispersed in the enriched sea water (Fig. 1). In the presence of 10^{-2} M azide or in the absence of shaking no dispersion was evident, even upon prolonged incubation. Figure 2 summarizes three parameters which accompanied the oil dispersion. The viable count increased about 100-fold during the first 24 hr and then declined steadily. More frequent measurements of the growth rate during the first day indicate an average doubling time of approximately 3 hr for the mixed culture under the conditions employed. The *p*H decreased from 7.5 to 5.0 during the first 2 days and then remained constant for the duration of the experiment. Most of the drop occurred during the second day, a time when there was no net increase in the number of viable cells. In certain other experiments in which inbuation was continued for longer periods, the *p*H rose slowly back to about 7.0. The last parameter to change was oil dispersion, which occurred only after a lag of 2 days. The oil emulsion was stable even in

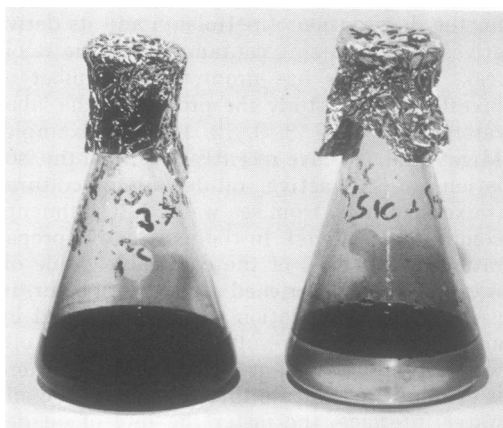

FIG. 1. *Microbial dispersion of crude oil. The flask on the right is a control containing 10^{-2} M azide.*

prolonged experiments where the pH rose to 7.0.

The data summarized in Fig. 3 demonstrate that the oil dispersion is a biological process requiring supplementation of the sea water. In the presence of azide there was no measurable oil dispersion and only a small drop in the pH. The small population of azide-resistant microorganisms which began to increase after a day were apparently unable to disperse the oil. It is possible that they were able to increase to 10^7 per ml at the expense of the 0.005% yeast extract in this medium. Without any supplementation of the sea water with ammonia, phosphate, and yeast extract, there was no oil dispersion and the microbial population was limited to 2×10^7 cells/ml. If the crude oil was filter sterilized and no inoculum was added, no changes in pH or oil dispersion occurred.

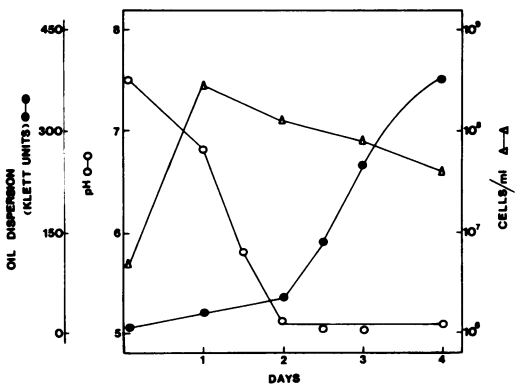

FIG. 2. *Changes in the pH, cell number, and dispersion of oil during incubation at 32 C. One hundred-milliliter flasks containing 30 ml of sterile sea water, 150 mg of crude oil, 0.057 mM K_2HPO_4, 7.6 mM $(NH_4)_2SO_4$, and 100 μg of yeast extract were inoculated with 0.1 ml of a 2-day mixed bacterial culture. See Materials and Methods for the preparation of the inoculum and the determinations of oil dispersion and viable count.*

Experiments in which the inoculum was supplied either by fresh sea water or crude oil rather than by the enrichment culture gave variable results. In some cases, oil dispersion was almost as rapid as with the inoculum from the enrichment culture; in other cases, there was no significant change in the oil for more than 2 weeks.

The results of a more extensive study of nitrogen and phosphorus requirements for microbial oil dispersion of crude oil indicated that optimum rates of oil dispersion were achieved when K_2HPO_4 and $(NH_4)_2SO_4$ were added to the sea water to final concentrations of 0.029 mM and 7.6 mM, respectively. These experiments were performed in the absence of added yeast extract since preliminary data indicated that, with adequate concentrations of ammonia and phosphate, there was no stimulation by yeast extract. Oil dispersion was never observed unless the sea water was supplemented with both a source of nitrogen and phosphorus. Ammonium chloride could substitute for ammonium sulfate.

Dispersion of crude oil by pure cultures. When the enrichment culture was streaked onto supplemented oil agar plates, several different colony types were observed. The eight most frequent types were obtained in pure culture by standard bacteriological techniques and screened for their ability to disperse oil. It is interesting to note that all of the isolates could form colonies both on supplemented oil agar and nutrient agar. However, only one of the isolates, subsequently referred to as RAG-1, brought about a significant dispersion of crude oil. Although the other seven isolates could grow on supplemented oil and several of them could lower the pH to 5 to 6, they neither dispersed the oil nor stimulated the dispersion catalyzed by RAG-1.

RAG-1 has the following characteristics.

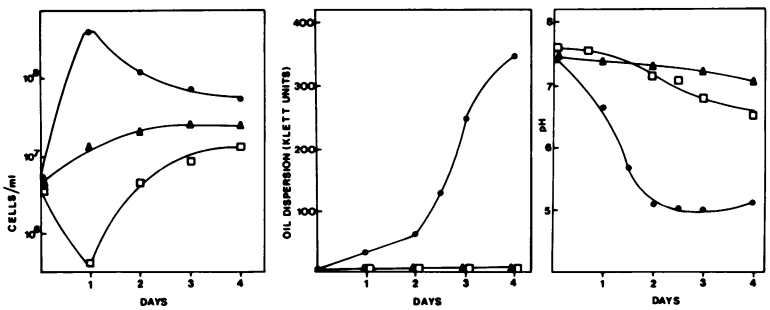

FIG. 3. *Parameters affecting the microbial utilization of crude oil. The conditions for the complete system were those described in Fig. 2. Experimental flasks: complete (●); plus 10^{-2} M azide (□); minus ammonia, phosphate, and yeast extract (△).*

During the exponential growth phase the cells appear mostly as irregular short rods, 0.9 to 1.2 by 1.5 to 3.0 μm. The cells occur often as V-shaped pairs, indicating snapping division. Occasionally the rods are slightly bent or swollen. Coccoid cells, approximately 1.2 μm in diameter, are characteristic of stationary phase cultures. The cocci are gram-positive; the rods are gram-negative.

Agar colonies: circular, glistening and smooth, up to 5.0 mm in diameter; gelatin is liquified; starch is not hydrolyzed; indole and H_2O_2 are not produced; nitrites are produced from nitrate only when the cells are grown in citrate medium containing KNO_3; urease is not produced; catalase-positive; aerobic; hemolysis of rabbit blood agar; citrate can serve as the sole carbon and energy source; no acid from glucose, cellulose, maltose, lactose, rhamnose, sucrose or mannitol; optimum temperature 30 to 35 C. These properties characterize RAG-1 as a member of the genus *Arthrobacter*.

Figure 4 shows the kinetics of cell growth, oil dispersion, and changes in the *p*H brought about by RAG-1. In general, the data are similar to those shown in Fig. 2 for the mixed culture. The major difference was that the lag in oil dispersion was reduced from 2 days to 1 day when a pure culture of RAG-1 was employed as an inoculum. If the inoculum was previously grown on nutrient broth instead of supplemented oil, the drop in *p*H, oil dispersion, and cell growth were all delayed approximately 1 day. The nitrogen and phosphorus requirements for RAG-1-catalyzed oil dispersion are shown in Table 1. Optimum emulsification was achieved when the sea water was supplemented with 0.029 mM K_2HPO_4 and 3.8 mM $(NH_4)_2SO_4$. Ammonium chloride and urea could also serve as sources of nitrogen.

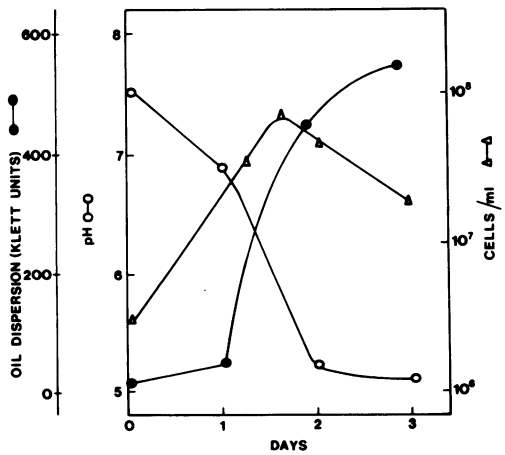

FIG. 4. *Changes in the pH, cell number, and dispersion of oil during incubation at 32 C with RAG-1. Thirty milliliters of sterile sea water containing 125 mg of filter-sterilized crude oil. 0.057 mM K_2HPO_4 and 7.6 mM $(NH_4)_2SO_4$ were inoculated with 1 ml of RAG-1 and incubated in a 250-ml flask with shaking. The inoculum was previously grown in the supplemented oil medium.*

Since oil dispersion in the unbuffered system was invariably accompanied by a drop in the *p*H and a decline in the viable count, it seemed worthwhile to measure oil dispersion under conditions of *p*H control (Table 2). The data clearly indicate that oil dispersion occurs at least as well from *p*H 6.8 to 7.4 as at *p*H 5.1. Furthermore, some of the pure cultures isolated were able to multiply on crude oil and lower the *p*H to 5 to 6, but were unable to disperse the oil. Thus, acidic conditions are neither necessary nor sufficient for oil dispersion.

Preliminary experiments indicate that RAG-1 catalyzed dispersion is brought about by the production of a dispersing agent(s), which can be found in the supernatant fraction. Following separation of the cells from the growth medium by centrifugation at 15,000 × *g* for 20 min, all of the dispersing power was in the supernatant fraction. When crude oil was added to the supernatant fraction, oil dispersion was rapid (within 60 min) and was unaffected by the presence of 10^{-2} M azide. Similarly, the supernatant fractions obtained following growth of RAG-1 on sea water with pentadecane, hexadecane, heptadecane, or succinate as sole sources of added carbon were capable of rapidly emulsifying crude oil.

TABLE 1. *Effect of ammonia and phosphate concentrations on RAG-1 dispersion of crude oil*

Supplement[a]		Oil dispersion[b] (Klett units)	*p*H[b]
K_2HPO_4 (mM)	$(NH_4)_2SO_4$ (mM)		
0.057	0	1	7.8
0.057	0.76	340	7.2
0.057	3.8	>1000	5.3
0.057	7.6	>1000	4.9
0.057	15.2	750	5.0
0	7.6	242	7.2
0.006	7.6	620	6.6
0.029	7.6	>1000	5.0
0.114	7.6	950	4.8

[a] In addition to supplements, each 250-ml flask contained 30 ml of sterile sea water, 125 mg of filter-sterilized oil and 0.15 ml of a 1-day-old inoculum of RAG-1. The flasks were incubated at 32 C with reciprocal shaking.

[b] Measurements were performed after 4 days; the technique for estimating oil dispersion is described in Materials and Methods.

Factors affecting the dispersion of crude oil in sea water are summarized in Table 3. The conditions employed in this experiment more closely approximate the "natural" situation since neither the sea water nor crude oil were sterilized. Without supplementation with ammonia and phosphate, no dispersion was recorded even if the flasks were inoculated with RAG-1. The most rapid and reproducible dispersion of oil took place when the flasks were supplemented with ammonia, phosphate, and an inoculum of RAG-1. However, in some experiments the sea water or crude oil, or both, provided a sufficient inoculum of microorganisms so that addition of RAG-1 was unnecessary. Organisms capable of dispersing crude oil and resembling RAG-1 morphologically were occasionally isolated from nonsterilized crude oil in the presence of sterilized sea water supplemented with ammonia and phosphate.

TABLE 2. *The dispersion of crude oil by RAG-1 under conditions of pH control*

pH control[a]	pH on day 5	Viable cells/ml		Oil dispersion (Klett units)	
		Day 2	Day 5	Day 2	Day 5
None	5.1	3×10^7	4×10^6	250	920
0.01 M Tris, pH 8.0	7.4	3×10^8	3×10^7	345	970
Periodic addition of KOH	6.8	2×10^8	3×10^7	520	920

[a] The experimental conditions are those described in Fig. 4.

TABLE 3. *Factors affecting the dispersion of crude oil in sea water*

Supplement[a]	Oil dispersion (Klett units)		pH	
	1 Day	4 Days	1 Day	4 Days
1. None	0	0	7.8	7.8
2. $K_2HPO_4 + (NH_4)_2SO_4$	123	335	7.4	5.9
3. RAG-1	12	5	7.8	7.8
4. RAG-1 + K_2HPO_4 + $(NH_4)_2SO_4$	210	490	7.1	5.9
5. RAG-1 + K_2HPO_4 + $(NH_4)_2SO_4$ + 10^{-2} M azide	0	0	7.5	7.4
6. As in 4 except oil and sea water sterilized	0	510	7.4	6.2

[a] Each 250-ml flask contained: 30 ml of nonsterile sea water and 125 mg of nonsterile crude oil. Flasks were incubated at 32 C with reciprocal shaking. K_2HPO_4 and $(NH_4)_2SO_4$ when added were at 0.057 mM and 7.6 mM, respectively. RAG-1 when added was a 0.3 ml inoculum of a 24-hr culture.

Growth and oil conversion by RAG-1 as a function of oil concentration is shown in Table 4. Over the range 0.05 to 1.0 mg of crude oil per ml, the cell increase was directly proportional to the quantity of crude oil added to the medium. Within this linear range, 1.0 mg of crude oil yielded 9×10^7 cells and approximately 65% of the oil was converted into a nonbenzene extractable form. The average doubling time was approximately 170 min under the conditions employed for this experiment.

DISCUSSION

The emulsification of crude oil by bacteria is absolutely dependent on exogenous sources of nitrogen and phosphorus, in agreement with the observations of other investigators (1, 7, 11). This requirement for nitrogen and phosphorus was observed regardless of whether an enriched population or a pure strain was used to catalyze the emulsification. The dependence on an exogenous source of nitrogen and phosphorus was found even when fresh nonsterilized sea water was used as a source of inoculum, and most likely accounts for the very slow breakdown of oil in the open sea.

Although a number of investigators have studied the utilization of crude oil catalyzed by enrichment cultures, attempts to reproduce the process by pure cultures were unsuccessful

TABLE 4. *Growth and oil conversion by RAG-1 as a function of oil concentration*

Oil supplement[a] (mg/300 ml)	Cells[b]/ml	Cells/mg of oil	Benzene-extractable oil remaining (mg)[b]	Oil conversion[c] (%)
1. 10	1.9×10^6	5.7×10^7	3.2	64
2. 20	6.0×10^6	9.0×10^7	5.9	67
3. 40	2.1×10^7	15.6×10^7	6.8	81
4. 100	3.0×10^7	9.0×10^7	31.8	66
5. 200	6.0×10^7	9.0×10^7	65.5	64
6. 400	9.5×10^7	7.1×10^7	149.2	61

[a] Each 2,000-ml flask contained: 300 ml of sea water, 0.057 mM K_2HPO_4, 7.6 mM $(NH_4)_2SO_4$, and varying quantities of crude oil. After inoculation with 0.2 ml of a suspension of 10^7 washed cells/ml, the flasks were incubated at 32 C with reciprocal shaking for 4 days.

[b] Measurements were performed after 4 days; the benzene extraction technique is described in Materials and Methods.

[c] Control flasks, treated like the experimental flasks except lacking bacteria, indicated that 90% of the added oil was extractable after 4 days by the techniques employed. The values reported are corrected accordingly. For example, in experiment 1: % oil conversion = (9.0 − 3.2/9.0) × 100.

(8, 12, 14). The use of pure cultures in the study of microbial degradation of crude oil provides technical advantages by eliminating the ambiguity associated with constantly fluctuating populations. In the present study, eight different colony types capable of growing on crude oil were isolated. It is of interest to note that of these eight only RAG-1 catalyzed emulsification. This bacterium which has been tentatively identified as an *Arthrobacter* species will be the subject of a subsequent communication. RAG-1-induced dispersion essentially paralleled that observed with the enrichment culture, although maximum emulsion was achieved 24 hr earlier with the pure culture. This difference may be the result of the presence of other bacterial populations in the enrichment culture which inhibit the production or action of an emulsifying agent (P. Guire et al., Fed. Proc. *31*, p. 1482, 1972).

Two possible explanations for the relatively low yield of RAG-1 on crude oil (10^8/ml on 0.1% oil, Table 4) are as follows: (i) RAG-1 utilizes only a small fraction of the components found in crude oil, or (ii) RAG-1 utilizes most of the components in crude oil but only to a limited extent. That is, it performs only a partial oxidation. The fact that most of the oil is converted to a form which is no longer extractable with benzene supports the second explanation. Fractionation and chemical identification of the products of RAG-1 grown on crude oil and pure hydrocarbons is presently under investigation in order to obtain a more definitive answer to this question. As part of this study, the purified fractions will be examined for their ability to disperse oil.

Thus far, the enrichment techniques have permitted the isolation of mixed and pure cultures which will disperse but only partially degrade oil in the laboratory. In this regard, Miget et al. (8) have stressed the need to extend studies with bacteria to an open system more accurately reflecting conditions in the sea. In an open system the accumulation of easily diffusible toxic products may be eliminated and RAG-1 might be able to utilize the oil more efficiently. The fact that a more rapid dispersion took place in buffered sea water (Table 2) provides some evidence for this suggestion. Alternatively, the breakdown of crude oil may not be as effective in an open system due to the dilution of factors required for growth or degradation, or both, by the bacteria. In this regard, the strict requirement for nitrogen and phosphorus supplementation suggests that the utilization of microorganisms in the treatment of oil pollution must be limited to situations in which the polluting oil is confined.

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