APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1979, p. 409-413
0099-2240/79/03-0409/05$02.00/0

Vol. 37, No. 3

Emulsifier of *Arthrobacter* RAG-1: Specificity of Hydrocarbon Substrate

E. ROSENBERG,[1]* A. PERRY,[1] D. T. GIBSON,[2] AND D. L. GUTNICK[1]

Department of Microbiology, George S. Wise Center for the Life Sciences, Tel Aviv University, Tel Aviv, Israel,[1] and Department of Microbiology, University of Texas, Austin, Texas 78712[2]

Received for publication 29 December 1978

The purified extracellular emulsifying factor produced by *Arthrobacter* RAG-1 (EF-RAG) emulsified light petroleum oil, diesel oil, and a variety of crude oils and gas oils. Although kerosine and gasoline were emulsified poorly by EF-RAG, they were converted into good substrates for emulsification by addition of aromatic compounds, such as 2-methylnaphthalene. Neither aromatic nor aliphatic fractions of crude oil were emulsified by EF-RAG; however, mixtures containing both fractions were emulsified. Pure aliphatic or aromatic hydrocarbons were emulsified poorly by EF-RAG. Binary mixtures containing an aliphatic and an aromatic hydrocarbon, however, were excellent substrates for EF-RAG-induced emulsification. Of a variety of alkylcyclohexane and alkylbenzene derivatives tested, only hexyl- or heptylbenzene and octyl- or decylcyclohexane were effectively emulsified by EF-RAG. These data indicate that for EF-RAG to induce emulsification of hydrocarbons in water, the hydrocarbon substrate must contain both aliphatic and cyclic components. With binary mixtures of methylnaphthalene and hexadecane, maximum emulsion was obtained with 25% hexadecane.

Emulsifiers and surfactants are classified as anionic, cationic, or nonionic. Also, so-called HLB (hydrophile-lipophile balance) numbers are used advantageously as initial guides in commercial emulsion formulation (14). Little attention, however, has been paid to the possibility that emulsifiers with similar HLB numbers may interact differently with hydrocarbon substrates. Biologically produced polymers often exhibit specificities not seen in chemically synthesized materials. In this regard it was of interest to extend the characterization of the extracellular *Arthrobacter* emulsifier, referred to as EF-RAG. In an accompanying paper (13) a convenient and sensitive assay for measuring emulsification was described. This procedure allowed for the detailed investigation of hydrocarbon substrate specificity presented in this report.

MATERIALS AND METHODS

Petroleum products. Agha Jari, Rostam, and Darius crude oils and bunker C fuel oil were obtained from the Ashkelon-Eilat Pipeline Co., Israel. The chemical and physical properties of these paraffinic Iranian crude oils have been reported (2, 4). Kerosine, diesel, and gas oil fractions were obtained from the Haifa Refinery, Haifa, Israel. All cyclohexane derivatives were obtained from Chemical Samples Inc., Columbus, Ohio. Octyl-, nonyl-, and decylcyclohexanes were redistilled. Olefin-free hexadecane (>99% purity) was obtained from Fluka Chemical Co., Switzerland. *n*-Alkylbenzenes and *n*-octacosane were products of Eastman Chemicals. Phenanthrene and anthracene were obtained from Baker Chemicals. Adamantane, 1-methylnaphthalene, 2-methylnaphthalene, hexatriacontane, nondecane, 3-phenyltoluene, dicyclohexane, *p*-diisopropylbenzene, and 2,6,10,14-tetramethylpentadecane were obtained from Aldrich Chemicals. Light paraffinic oil (density 0.85 g/cm^3) was obtained from British Drug Houses Ltd., Poole, England. The remaining hydrocarbons were obtained from Phillips Petroleum Co.

Column fractionation of crude oil. Agha Jari crude oil, fractionated by the procedure of Jobson et al. (8), yielded 1.7% asphaltane, 59.5% saturate, 14% aromatic, and 23.2% polar aromatic fractions.

Bacterial emulsifying agent (EF-RAG). The extracellular emulsifying factor of *Arthrobacter* RAG-1 was prepared by two procedures as described in detail previously (13). EF-RAG(HD) was prepared by the heptane extraction procedure from a hexadecane-grown culture; heptane was used to remove residual growth substrate. EF-RAG(UET) was prepared by ammonium sulfate fractionation of an ethanol-grown culture.

Determination of hydrocarbon emulsification. Unless stated otherwise, emulsion formation was measured in 125-ml rubber-stoppered flasks, containing 5 ml of filtered seawater and varying concentrations of EF-RAG and hydrocarbon. Flasks were agitated by gyratory shaking (280 rpm) in a New Brunswick G24 incubator shaker for 2 h at 25°C. Contents

of the flask were then transferred to Klett tubes for measurement of turbidity in a Klett-Summerson colorimeter fitted with a green filter. Readings were taken after standing undisturbed for 10 min. Appropriate dilutions were made in water so that the final reading was between 30 and 150 Klett units. Reported values for Klett units are final readings times the dilutions. Controls lacking either EF-RAG or hydrocarbon yielded readings of less than 5 Klett units. All values reported are the average of at least three determinations. The average standard deviation of a single measurement between 30 and 1,000 Klett units was 7% of the corrected reading.

RESULTS

Emulsification of petroleum fractions by EF-RAG. The ability of the extracellular emulsifying agent of *Arthrobacter* sp. RAG-1, EF-RAG, to emulsify crude oil and fractions of crude oil is summarized in Table 1. All crude oils tested were emulsified by EF-RAG. In addition to those shown in Table 1, various crude oils from Alaska, Louisiana, and Texas were emulsified by EF-RAG. In general, better emulsions were obtained with reciprocal than with gyratory shaking. Gas oil was a better substrate for EF-RAG-induced emulsification than kerosine. In fact, emulsions of gas oil were as stable as crude-oil emulsions; the major reason for the higher Klett readings of crude-oil emulsions than gas oil emulsions was the dark color of crude oil compared to gas oil. Diesel light petroleum (density, 0.83 g/cm^3) and yielded emulsions similar to gas oil, whereas gasoline behaved like kerosine with regard to EF-RAG. Emulsions of kerosine and gasoline were unstable. Bunker C fuel oil was emulsified by EF-RAG(UET) but only poorly by EF-RAG(HD).

TABLE 1. *Emulsification of petroleum fractions by EF-RAG*

Petroleum fraction (8 mg/ml)	Emulsifier (50 μg/ml)	Emulsion (Klett units)
Crude oils		
Darius	EF-RAG(UET)	650, 1,090[a]
Agha Jari	EF-RAG(UET)	720, 950[a]
Agha Jari	EF-RAG(HD)	780
Rostam	EF-RAG(HD)	758
Gas oils		
Darius	EF-RAG(UET)	300, 880[a]
Gach-Saran	EF-RAG(UET)	500
Belayim marine	EF-RAG(UET)	100
Agha Jari	EF-RAG(UET)	195, 840[a]
Agha Jari	EF-RAG(HD)	420
Kerosines		
Darius	EF-RAG(UET)	42, 160[a]
Belayim marine	EF-RAG(UET)	35
Agha Jari	EF-RAG(UET)	41, 110[a]
Agha Jari	EF-RAG(HD)	125
Miscellaneous		
Diesel oil	EF-RAG(UET)	290
Diesel oil	EF-RAG(HD)	490[a]
Bunker C fuel oil	EF-RAG(UET)	680[a]
Bunker C fuel oil	EF-RAG(HD)	35[a]
Light petroleum oil	EF-RAG(HD)	218[a]
Gasoline (83 octane)	EF-RAG(UET)	89[a]

[a] Reciprocal shaking (150 strokes per min) instead of gyratory shaking.

Emulsification of pure hydrocarbons by EF-RAG. Straight- and branched-chain aliphatic hydrocarbons (0.05 ml/7.5-ml assay volume) from heptane to octadecane were emulsified only to a slight extent by EF-RAG (100 μg/ml). The branched-chain alkanes tested were 2,2,5-trimethylhexane, 2-methyldecane, 2,6-dimethyldecane, and 2,6-dimethylunidecane. Octadecane was incubated at 37°C to avoid solidification. Increasing or decreasing the hydrocarbon concentration by a factor of five did not improve emulsification. Pentane and hexane also were not emulsified effectively; however, quantitative data for these two paraffins were not obtained because of extensive evaporation during incubation. The solid hydrocarbons, nonadecane, *n*-octacosane, and hexatriacontane, were not dispersed by EF-RAG.

Experimental results on the emulsification of *n*-alkyl cyclohexane derivatives from propylcyclohexane to tridecylcyclohexane by EF-RAG are summarized in Table 2. Two peaks of activity were observed, corresponding to octylcyclohexane and decyl cyclohexane. The data for octyl-, nonyl-, and decylcyclohexanes were obtained from redistilled materials which contained no ultraviolet-absorbing impurities. Concentrations of octyl- and decylcyclohexane as low as 5 mg/ml were rapidly and completely emulsified by 50 μg of EF-RAG per ml. Nonylcyclohexane did not contain any apparent inhibitors of emulsification since mixtures of octyl- and nonylcyclohexane were emulsified to about the same extent as octylcyclohexane alone. Bicyclohexane and decalin were not emulsified significantly.

Data on the emulsification of *n*-alkylbenzene derivatives by EF-RAG are also summarized in Table 2. Maximum activity was obtained with hexyl- and heptylbenzenes. The total number of carbon atoms in the side chains may be more crucial than the chain length since *p*-diisopropylbenzene behaved like hexyl-benzene. The low-molecular-weight benzene derivatives toluene, *p*-xylene, ethylbenzene, and 1,2,3,4-tetramethylbenzene were not emulsified significantly. Aromatic compounds containing more than one ring, naphthalene, biphenyl, phenanthrene, anthracene, 3-phenyltoluene, 1-methylnaphthalene, and 2-methylnaphthalene, were not emulsified by EF-RAG.

Emulsification of mixtures of pure hydrocarbons. Table 2 summarizes a number of ex-

TABLE 2. *Emulsification of mixtures of aliphatic, aromatic, and cyclic hydrocarbons by EF-RAG*

Hydrocarbon[a]	Emulsion (Klett units)		
	No addition	Plus hexadecane	Plus 1-methylnaphthalene
Aliphatics			
Decane	15	41	185
Tetradecane	13	50	216
Hexadecane	20	31	284
Nonadecane	0 (solid)	79	285
2,2,5-Trimethylhexane	0	34	89
2,6-Dimethylunadecane	0	2	105
Aromatics			
Biphenyl	0 (solid)	123[b]	19[b]
Naphthalene	0 (solid)	96[b]	26[b]
Phenanthrene	0 (solid)	61[b]	36[b]
Toluene	22	97	4
3-Phenyl toluene	0	157	0
1-Methylnaphthalene	0	284	0
2-Methylnaphthalene	0	244	0
p-Xylene	22	75	15
Ethylbenzene	9	117	21
Propylbenzene	9	90	23
Pentylbenzene	4	197	85
Hexylbenzene	98	188	165
p-Diisopropylbenzene	96	299	192
Heptylbenzene	105	82	186
Decylbenzene	38	31	49
Pentadecylbenzene	21	0	5
1,2,3,4-Tetramethylbenzene	28	35	9
Cycloparaffins			
Ethylcyclohexane	8	81	43
Propylcyclohexane	3	81	64
Butylcyclohexane	0	111	57
Hexylcyclohexane	5	9	116
Heptylcyclohexane	1	32	131
Octylcyclohexane	109	151	175
Nonylcyclohexane	0	0	249
Decylcyclohexane	79	192	171
Dodecylcyclohexane	5	0	72
Decalin	0	15	17
Dicyclohexane	14	201	39

[a] Experiments were performed using 50 μg of EF-RAG(HD) per ml and 0.025 ml of each hydrocarbon (20 mg for solids).

[b] For solubility reasons, 0.05-ml solutions containing 10+ biphenyl, 10% naphthalene, and 5% phenanthrene in hexadecane or 1-methylnaphthalene were used.

periments in which EF-RAG-induced emulsification of aliphatic, aromatic, and cyclic hydrocarbons was measured in the presence of hexadecane or 1-methylnaphthalene. Although neither the aliphatic compounds (0 to 20 Klett units) nor 1-methylnaphthalene (0 Klett units) were emulsified by themselves, all mixtures containing the aromatic compound and one of the aliphatic hydrocarbons (89 to 285 Klett units) were excellent substrates for EF-RAG emulsification. The ability of aromatic compounds to stimulate emulsification of aliphatics was not limited to 1-methylnaphthalene, but also occurred with toluene, *p*-xylene, 3-phenyltoluene, and 2-methylnaphthalene. Addition of hexadecane to the aliphatic compounds did not stimulate emulsification; that is, only an additive effect was observed. The minor exception to this finding was nonadecane, which became liquid when mixed with hexadecane.

As mentioned above, the only aromatic compounds that served as substrate for emulsification by EF-RAG were alkylbenzene derivatives containing six or seven carbon atoms in the side chain(s). Aromatic compounds containing less than six carbon atoms on the side chain were converted into good substrates for emulsification by addition of hexadecane. Hexylbenzene and diisopropyl-benzene were converted into even better substrates for emulsification by addition of hexadecane. On the other hand, heptyl-, decyl-, and pentadecylbenzene were not emulsified better in the presence of hexadecane than by themselves. The emulsification of alkylbenzene derivatives containing side chains of five or more carbon atoms was enhanced by 1-methylnaphthalene. 1,2,3,4-Tetramethylbenzene was poorly emulsified by EF-RAG even in the presence of hexadecane or 1-methylnaphthalene. With few exceptions, cycloparaffin derivatives were converted into better substrates for EF-RAG-mediated emulsification by addition of either hexadecane or 1-methylnaphthalene. In general, the emulsification of cyclohexane derivatives with short side chains (e.g., ethylcyclohexane) were enhanced more efficiently with aliphatic than with aromatic compounds, whereas derivatives with long side chains (e.g., dodecylcyclohexane) formed better emulsions in the presence of 1-methylnaphthalene than in the presence of hexadecane. Dicyclohexane behaved like an aromatic compound in that it was emulsified by EF-RAG in the presence of hexadecane but not in the presence of 1-methylnaphthalene. The fused dicyclic compound decalin was not emulsified by EF-RAG even by addition of hexadecane or 1-methylnaphthalene.

EF-RAG-induced emulsion formation as a function of the relative concentrations of aliphatic (hexadecane) and aromatic (methylnaphthalene) compounds is shown in Fig. 1. Using either 1-methylnaphthalene or 2-methylnaphthalene, maximum emulsion was obtained with 25% hexadecane (vol/vol). Over 50% maximum emulsion was obtained with ratios of hexadecane to methylnaphthalene of from 4:1 to 1:6. An identical experiment using decane in place of hexadecane yielded similar curves except that the peak of emulsion activity was obtained with 33% (vol/vol) decane.

Effect of addition of aliphatic and aromatic compounds on emulsification of pe-

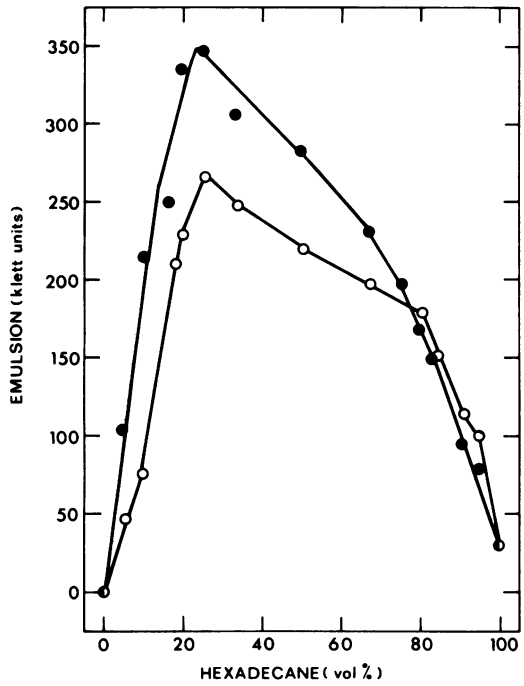

FIG. 1. *Emulsification of mixtures of hexadecane and methylnaphthalene by EF-RAG(HD). Experiments were performed using 50 μg of EF-RAG per ml and 0.05 ml of various mixtures of hexadecane and 1-methylnaphthalene* (●) *or hexadecane and 2-methylnaphthalene* (○).

troleum fractions by EF-RAG. The results shown in Table 2 and Fig. 1 suggest that the ability of EF-RAG to emulsify hydrocarbons depends on the relative concentrations of aliphatic, cyclic, and aromatic components. Thus, it was interesting to examine whether or not addition of hexadecane or methylnaphthalene could enhance EF-RAG-induced emulsification of petroleum fractions (Table 3). The ability of EF-RAG to emulsify both kerosine and gasoline was enhanced greatly by 2-methylnaphthalene but not by hexadecane. Addition of even one part of the aromatic compounds to ten parts of gasoline or kerosine resulted in a much improved substrate for emulsification. The requirement for both aliphatic and aromatic constituents was further supported by studying emulsification of column-fractionated crude oil. Although crude oil itself was emulsified by EF-RAG, none of the fractions were good substrates. However, mixtures containing one fraction rich in aliphatics (fraction 1) and the other rich in aromatics (fraction 2 or 3) were efficiently emulsified.

DISCUSSION

To our knowledge this represents the first detailed investigation of hydrocarbon substrate specificity of a bioemulsifier. The data indicate that for EF-RAG to emulsify hydrocarbon efficiently in water, the hydrocarbon substrate must contain both aliphatic and cyclic components. The aliphatic chain could be either straight or branched. Cyclic components that were tested and shown to be effective (mixed with hexadecane) included both aromatic compounds (biphenyl, naphthalene, phenanthrene, 3-phenyl toluene as well as several benzene and naphthalene derivatives) and nonaromatic compounds (dicyclohexane and cyclohexane derivatives).

Although EF-RAG can emulsify certain pure aromatic or cyclohexane derivatives which contain alkyl side chains of 6 to 10 carbon atoms, it is unlikely that these compounds contribute significantly to the ability of EF-RAG to emulsify crude oil and gas oil since ring compounds with side chains of more than five carbon atoms are rare in crude oils (3, 16). More likely, it is the presence of appropriate mixtures of alkyl and cyclic compounds in crude oil and certain fractions of crude oil that allow for EF-RAG-induced emulsion formation. This is consistent with the following data. (i) Fractionation of crude oil according to polarity rather than boiling point led to inactive fractions (Table 3); mixtures containing an aromatic fraction and an aliphatic fraction regained activity. (ii) Gasoline and ker-

TABLE 3. *Emulsification of mixtures of petroleum fractions and pure hydrocarbons by EF-RAG*[a]

Petroleum fraction	Addition[b]	Emulsion (Klett units)
Kerosine	None	190
	Hexadecane	68
	2-Methylnaphthalene	1,050
Gasoline	None	115
	Hexadecane	230
	2-Methylnaphthalene	1,100
Agha Jari		
Fraction 1	None	130
Fraction 2	None	60
Fraction 3	None	105
Fraction 1	Fraction 2	1,050
Fraction 1	Fraction 3	1,500
Fraction 2	Fraction 3	80

[a] Experiments were performed as described in the text with reciprocal shaking, using 50 μg of EF-RAG(UET) per ml and 8 mg of total substrate (petroleum fraction plus addition) per ml.

[b] The mixtures were 1:1 (vol/vol).

[c] Agha Jari crude oil was fractionated by the procedure of Jobson et al. (8). Fractions 1, 2, and 3 correspond to the aliphatic (saturates), aromatic, and polar aromatic fractions, respectively.

osine, which are relatively poor in aromatic content, could be converted into good substrates for emulsion by addition of 2-methylnaphthalene.

Knowledge of the detailed substrate specificity of an emulsifier has obvious applied value. In the case of the *Arthrobacter* emulsifier, not only does it allow one to predict from its chemical structure whether or not a particular compound will be emulsified by EF-RAG, but it also suggests which addition (i.e., aliphatic or cyclic compounds) will enhance emulsion formation.

At present, the natural role of bioemulsifiers (5, 7, 15, 17–19) is not clear. It has been suggested that bioemulsifiers and biosurfactants may function to enhance either pseudosolubilization of hydrocarbon (12) or direct contact between microorganisms and hydrocarbon substrate (6, 9, 11). Fermentation studies have demonstrated that growth rate can be limited by the interfacial surface area between water and oil (10). When the surface area becomes limiting, biomass increases arithmetically rather than exponentially (1). However, it has been pointed out that these studies were conducted at high cell masses, with forced aeration, vigorous mechanical agitation, and under optimum nutritional conditions, and, as such, may not be relevant to cell-oil interaction as it occurs in nature (4).

LITERATURE CITED

1. **Erickson, L. E., and T. Nakahara.** 1975. Growth in cultures with two liquid phases: hydrocarbon uptake and transport. Proc. Biochem. **10**:9–13.
2. **Ferrero, E. P., and D. T. Nidiols.** 1972. Analyses of 169 crude oils from 122 foreign oil fields. p. 53. Bureau of Mines Information Circular no. 8542. U.S. Department of the Interior, Washington, D.C.
3. **Giger, W., and M. Blumer.** 1974. Polycyclic aromatic hydrocarbons in the environment: isolation and characterization by chromatography, visible, ultraviolet and mass spectrometry. Anal. Chem. **46**:1663–1671.
4. **Gutnick, D. L., and E. Rosenberg.** 1977. Oil tankers and pollution: a microbiological approach. Annu. Rev. Microbiol. **31**:379–396.
5. **Holdom, R. S., and A. G. Turner.** 1969. Growth of *Mycobacterium rhodochrous* on n-decane: a new growth factor and emulsifying agent. J. Appl. Bacteriol. **34**:448–456.
6. **Horowitz, A., D. L. Gutnick, and E. Rosenberg.** 1975. Sequential growth of bacteria on crude oil. Appl. Microbiol. **30**:10–19.
7. **Itoh, S., and T. Suzuki.** 1972. Effect of rhamnolipids on growth of *Pseudomonas aeruginosa* mutant deficient in n-paraffin-utilizing ability. Agric. Biol. Chem. **36**: 2233.
8. **Jobson, A., F. D. Cook, and D. W. S. Westlake.** 1972. Microbial utilization of crude oil. Appl. Microbiol. **23**: 1082–1089.
9. **Kaeppeli, O., and A. Fiechter.** 1976. Mode of interaction between *Candida* and hydrocarbon. Biotechnol. Bioeng. **18**:967–974.
10. **Katinger, H. W. D.** 1973. Influence of interfacial area and nonutilizable hydrocarbons on growth kinetics of *Candida* Sp in hydrocarbon fermentations. Biotechnol. Bioeng. **4**:485–505.
11. **Kennedy, R. S., W. R. Finnerty, K. Sudarsanan, and R. A. Young.** 1975. Microbial assimilation of hydrocarbons. I. The fine-structure of a hydrocarbon oxidizing *Acinetobacter* Sp. Arch. Microbiol. **102**:75–83.
12. **Nakahara, T., L. E. Erickson, and J. R. Gutierrez.** 1977. Characteristics of hydrocarbon uptake in cultures with two liquid phases. Biotechnol. Bioeng. **19**:9–25.
13. **Rosenberg, E., A. Zuckerberg, H. Rubinowitz, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. **37**:402–408.
14. **Shaw, D. J.** 1970. Introduction to colloid and surface chemistry, p. 210–211. Butterworth & Co., London.
15. **Uguchi, T., I. Takeda, and H. Ohsawa.** 1969. Emulsifying factor of hydrocarbon produced by a hydrocarbon-assimilating yeast. Agric. Biol. Chem. **33**:1657–1658.
16. **Whitehead, W. L., and I. A. Breger.** 1963. Geochemistry of petroleum, p. 248–332. *In* I. A. Breger (ed.), Organic geochemistry. Pergamon Press, New York.
17. **Zajic, J. E., H. Guignard, and D. F. Gerson.** 1977. Emulsifying and surface active agents from *Corynebacterium hydrocarboclastus*. Biotechnol. Bioeng. **19**: 1285–1301.
18. **Zajic, J. E., H. Guignard, and D. F. Gerson.** 1977. Properties and biodegradation of a bioemulsifier from *Corynebacterium hydrocarboclastus*. Biotechnol. Bioeng. **19**:1303–1320.
19. **Zajic, J. E., and C. J. Panchal.** 1976. Bioemulsifiers. CRC Crit. Rev. Microbiol. **5**:39–66.