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Production of extracellular emulsifying agent by *Pseudomonas aeruginosa* UG1

Cynthia G. MacElwee, Hung Lee, and Jack T. Trevors

Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada

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SUMMARY

Twenty-three bacterial strains were isolated from oil-contaminated soil samples. Of these, 20 displayed some ability to effect oil dispersion and they were screened quantitatively for the ability to emulsify 0.5% (v/v) reference oil. One strain, identified as *Pseudomonas aeruginosa* UG1, produced extracellular material that emulsified reference oil, hexadecane and 2-methylnaphthalene at concentrations as high as 6% (v/v) in nutrient broth. Emulsification activity increased during a 10 day incubation period at 30°C. The activity was not influenced by pH over the range 5 to 9. The emulsifying agent was precipitated by cold ethanol. The highest emulsifying activity was detected in the extracellular fraction precipitated between 30 and 50% (v/v) ethanol. A linear relationship was observed between emulsifier concentration (mg/ml) and emulsifying activity. Genetic analysis showed that the *Pseudomonas aeruginosa* UG1 strain did not carry extrachromosomal plasmids, suggesting that the gene(s) coding for emulsifying activity was carried on the chromosome.

INTRODUCTION

The growth of bacterial and yeast cells on hydrocarbons is usually accomplished by emulsification of the water-insoluble hydrocarbons [4–7,9,11,15, 16,18–22,27]. For example, bacteria such as *Arthrobacter* [22], *Pseudomonas aeruginosa* [10], *Vibrionaceae* [17], *Corynebacterium hydrocarboclastus* [26,27], *Acinetobacter calcoaceticus* [12], *Bacillus subtilis* [7] and the yeast *Candida lipolytica* [3] produce emulsifying agents. *Pseudomonas spp.* are enzymatically versatile organisms involved in nutrient and mineral cycling in the aquatic and soil environments. They can also take up non-volatile liquid hydrocarbons, usually in submicron sizes [19]. Emulsification may facilitate solubilization by in-

Correspondence: Professors H. Lee or J.T. Trevors, Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

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creasing the effective surface area of the hydrocarbons [7, 25].

For many microorganisms, little is known about the characteristics of emulsifiers, their biological function(s) and mechanism(s) of action [22]. A recent review by Cooper [4] examined the different types of emulsifiers synthesized by selected microorganisms. Some surface-active emulsifiers are components of cell membranes [16], and therefore are an essential part of microbiological research. Microbial emulsifiers are also important from an applied point of view; as they are used in enhanced oil recovery [16] and oil emulsification [15]. Emulsifiers also have potential applications in foodprocessing, pharmacology, petroleum solubilization in storage tanks, and cleanup of sites contaminated with hydrocarbons [15].

This paper reports on the screening of bacterial strains isolated from oil-contaminated soil samples for the ability to emulsify a reference oil. Additional studies on a *Pseudomonas aeruginosa* UG1 strain, selected for its high emulsifying activity, were conducted to gain information on some factors influencing emulsifying activity.

MATERIALS AND METHODS

Chemicals

Virgin heavy atmospheric gas oil (reference oil) refined from a light Canadian crude oil was obtained from Esso Research Centre, Sarnia, Ontario, Canada. Hexadecane and 2-methylnaphthalene were obtained from Aldrich Chemical Company, Milwaukee, U.S.A. All other chemicals were of reagent grade.

Isolation/identification of bacteria and initial qualitative assessment of emulsifier production

Soil samples (2 g) collected from oil contaminated sites in Guelph, Ontario, Canada were added to 50 ml of Lennox Broth (LB, Acumedia, Baltimore, MD) amended with 0.5% (v/v) reference oil. The composition of LB was (in w/v): 1% casein digest, 0.5% yeast extract and 0.5% NaCl [14]. The cultures were placed in 500-ml baffled Bellco flasks and incubated at 30°C for 14 days at 220 rpm in a gyrotory shaker. In all subsequent experiments, cultures were incubated under these conditions unless otherwise stated. The cultures were sampled by streaking onto LB agar plates containing 0.5% (w/v) D-glucose. Individual colonies were picked and restreaked to obtain pure cultures. For short-term storage, cultures were streaked onto LB agar slants, and after growth, stored at 4°C. For long term storage, cultures were grown overnight in 5 ml LB containing 0.5% (w/v) D-glucose. A 0.9-ml aliquot was added to an Eppendorf microfuge tube containing 0.1 ml glycerol which had previously been sterilized by autoclaving at 121°C for 25 min. After mixing, cultures were stored at −20°C.

Emulsifier production by each bacterial isolate was initially assessed qualitatively using a modification of the method of Broderick and Cooney [2]. Briefly, a loopful of each isolate from an agar slant was transferred to 10 ml of LB broth in 125-ml flasks containing 0.5% (v/v) sterile reference oil (autoclaved at 121°C for 15 min) and incubated at 30°C. Flasks were incubated as previously described. Each culture was examined visually to assess the extent of oil dispersion above the growth medium. The cultures displaying the greatest dispersion in the shortest time were selected for further quantitative screening.

Isolated bacteria were stained for Gram reaction. Five high emulsifier producing isolates were tested for catalase and oxidase activities using standard procedures [8] followed by the API-NFT test (Analytab Products, Plainview, N.Y.) for tentative species identification. The *P. aeruginosa* culture used for plasmid analysis was grown in 10 ml LB broth containing 0.5% (w/v) D-glucose in 125-ml flasks shaken for 18 h at 30°C.

Quantitative screening methodology for emulsifier production

Cells from an agar slant were streaked onto a fresh LB agar plate containing 0.5% (w/v) D-glucose. After 16 to 18 h, a single colony was transferred to 50 ml LB broth containing 0.5% (v/v) reference oil and the culture was incubated in a 500-ml baffled Bellco flask. At 4 and 7 days, samples were

removed for determination of emulsifier activity. Emulsifier activity was initially determined using a modification of the method of Cirigliano and Carman [3]. A 0.5 ml aliquot of culture filtrate was obtained by centrifugation at 5000 × *g* for 10 min, followed by vacuum filtration through a glass fibre filter and a 0.80 μm cellulose acetate filter. The emulsifying agent would not pass through a filter smaller than 0.80 μm. This sample was added to 1.9 ml of 20 mM Tris buffer, pH 7.0, containing 10 mM $MgSO_4$ and 0.1 ml hexadecane/2-methylnaphthalene (1:1, v/v) mixture in a screw-capped glass test tube (16 × 125 mm; 17 ml capacity). A second and third set replaced the hexadecane/2-methylnaphthalene mixture with either hexadecane or the Tris-Mg^{2+} buffer. The latter served as the control. Tubes were closed tightly and shaken at 220 rpm for 1 h at 30°C. The turbidity (measure of oil dispersion) was measured spectrophotometrically at 540 nm in a Philips Pye Unicam PU 8610 spectrophotometer. It was found during the screening that the assay mixture yielded more consistent results if the tube was vortexed for 10 s and held for 30 min to allow good dispersion prior to measurement at 540 nm. This procedure was followed in all activity determinations following the screening. One unit of activity (U) is defined as the amount of emulsifier giving an absorbance of 1.0 at 540 nm.

Effect of substrates on emulsifier production

The effect of reference oil concentration on emulsifier production by a *Pseudomonas aeruginosa* strain isolated from soil as described above was studied using 2 or 6% (v/v) reference oil in 50 ml of LB broth. In addition, induction of emulsifier production was investigated using 2 or 6% hexadecane or a mixture of hexadecane/2-methylnaphthalene (1:1, v/v). In these experiments, each medium was inoculated with 0.1 ml of a 16-h culture grown in 5 ml LB broth containing 0.5% (w/v) D-glucose. The experiment was conducted in duplicate with two separate inocula. Samples were removed at intervals for 10 days for determination of protein, colony forming units (cfu) and emulsifier activity. Many culture supernatants from hexadecane cultures became extremely viscous after day 5, and it was necessary to dilute samples 2-fold with sterile distilled H_2O to facilititate centrifugation and collection of the supernatants. Due to a small sample volume, filtration was not possible after centrifugation.

Cell counts

Samples were serially diluted in sterile 0.85% (w/v) sodium chloride. A 0.1-ml aliquot of the 10^{-6}, 10^{-7} and 10^{-8} dilutions were plated in duplicate onto LB agar containing 0.5% (w/v) D-glucose. Plates were incubated for 24 to 48 h at 30°C until cfu appeared.

Protein determinations

One ml of culture was centrifuged at 6000 × *g* for 8 min in a 1.5-ml Eppendorf microfuge tube. The supernatant was discarded, and the pellet washed twice in 0.1 M potassium phosphate buffer, pH 7.0. The cells were resuspended in 0.5 ml of buffer and maintained at –20°C until analysis.

Cells were lysed by adding 55 μl 10 N NaOH, and heating at 100°C for 30 min. Protein content of the lysed cells was determined using the Bio-Rad protein dye-binding reagent (Bio-Rad, Mississauga, Canada). Bovine serum albumin (Sigma Chemical Co., St. Louis, U.S.A.) served as the standard. Samples were diluted appropriately to yield absorbance readings lower than 0.85.

Emulsifier precipitation

The emulsifier producing *P. aeruginosa* strain was inoculated into 100 ml of LB broth containing 4% (v/v) reference oil and incubated at 30°C for 8 days at 200 rpm. Cells were harvested by centrifugation at 10 000 × *g* for 20 min at 4°C using a Sorvall RC5B refrigerated centrifuge. The supernatant was collected, and subjected to fractional precipitation using ice cold ethanol. Initially ethanol was added to 20% (v/v) and the solution was kept at 4°C for 1 h, and then centrifuged at 10 000 × *g* for 20 min. The precipitated material was collected and placed in dialysis tubing (12 000 to 14 000 MW cut off). To the supernatant was added more ethanol to 30%, and then to 50% (v/v). The precipitation and dialysis procedures were repeated at 30% and 50% ethanol concentrations. Dialysis was performed against

3 l of ultrapure H_2O (17.6 megaohms) for 2 days at 4°C with four water changes. The tubing was then placed in 100% acetone to remove water from the dialysate and allowed to air dry. The resulting dried material was collected, weighed and redissoloved in ultrapure H_2O for determination of emulsifier activity. The fraction containing the highest activity was used to study the relationship between emulsifier concentration and emulsifier activity.

RESULTS AND DISCUSSION

Twenty-three bacterial strains were isolated from 2 oil-contaminated soil samples. On qualitative assessment, 20 strains showed some ability to disperse the reference oil over a 10 day period and they were selected for quantitative screening for emulsifier production. Among the selected strains, 18 exhibited a Gram-negative reaction. The remaining 2 were Gram-positive. All 20 isolates were rod shaped, and all grew well at 30°C. They also exhibited growth over a temperature range of 16 to 37°C. When cultured on LB agar, the Gram-negative rods produced small pinpoint colonies (1 to 2 mm in diameter) which turned blue-green after 48 h. The 2 Gram-positive isolates displayed either small pinpoint colonies or larger colonies with no apparent colour change, but retained a creamy colouration which did not change on prolonged incubation. All strains selected for quantitative screening exhibited the small, blue-green, pinpoint colony morphology.

During quantitative screening, emulsifying activities produced by the strains ranged from 0.04 to 2.0 U/ml supernatant for samples taken after 7 days of growth. An arbitrary value of 1.0 U/ml was used as the value to be exceeded when selecting for high emulsifier activity. Of the 20 isolates, 5 showed activities greater than 1.0 U/ml. All 5 isolates tested positive for catalase and oxidase activities, and according to the API identification scheme, belonged to the genus *Pseudomonas*. The strain which displayed the highest emulsifier activity (activity = 2.0 U/ml) was selected for all subsequent studies. It was identified as a *P. aeruginosa* strain and was designated as UG1.

The production of extracellular emulsifier activity by *P. aeruginosa* UG1 occurred gradually over the 10 day incubation period. (Tables 1 to 3). Emulsification of 6% (v/v) reference oil after 10 days was highly efficient (Table 1). When reference oil concentration was increased from 2 to 6%, higher

Table 1

Production of extracellular emulsifying activity by *P. aeruginosa* UG1 grown on reference oil

Time (days)	Oil (% v/v)	Emulsifying activity[a]		Log cfu/ml[b]
		(U/ml culture)	(U/mg cell protein)	
1	2	0.7	6.0	ND[c]
2		2.0	7.0	10
4		9.5	11	10
8		8.0	41	8.7
10		11	84	8.9
1	6	0.9	4.0	ND
2		4.5	11	ND
4		11	15	9.5
8		27	280	11
10		33	960	8.5
10	Control (no reference oil)	0.01	0.1	8.5

[a,b] Mean of two independent determinations. [c] Not determined.

Table 2

Production of extracellular emulsifying activity by *P. aeruginosa* UG1 grown on a hexadecane/2-methylnaphthalene (H/M) mixture

Time (days)	H/M (% v/v)	Emulsifying activity[a]		Log cfu/ml[b]
		(U/ml culture)	(U/mg cell protein)	
1	2	1.0	6.2	ND[c]
2		3.0	40	ND
4		2.6	100	8.0
8		6.9	330	8.0
10		21	560	8.5
1	6	1.5	9.3	ND
2		4.3	12	7.6
4		2.1	16	7.5
8		20	49	8.5
10		13	400	8.7
10	Control (no H/M mixture)	0.01	0.1	8.5

[a,b] Mean of two independent determinations. [c] Not determined.

emulsifier activity was generally observed over 10 days. This suggested that a relationship might exist between oil concentration and emulsifying activity. The highest activity (960 U/per mg protein) was observed after 10 days of growth in the presence of 6% (v/v) reference oil. Activities were expressed as U/ml culture or U/mg cell protein for comparison (Tables 1 to 3). Viable cfu were greater than Log 8 even after 10 days. In addition, the emulsifying activity was not altered when assayed over a pH range of 5 to 9 (data not shown).

The production of extracellular emulsifying ac-

Table 3

Production of extracellular emulsifying activity by *P. aeruginosa* UG1 grown on hexadecane

Time (days)	hexadecane (% v/v)	Emulsifying activity[a]		Log cfu/ml[b]
		(U/ml culture)	(U/mg cell protein)	
1	2	5.2	14	ND
2		ND[c]	ND	11
4		13	27	11
8		46	76	11
10		40	130	11
1	6	1.6	5.1	ND
2		3.3	8.3	10
4		15	36	10
8		80	220	11
10		36	170	11
10	Control (no hexadecane)	0.01	0.1	8.5

[a,b] Mean of two independent determinations. [c] Not determined.

tivity was also assessed when *P. aeruginosa* UG1 was cultured in a hexadecane/2-methylnaphthalene mixture (Table 2) or hexadecane (Table 3). Production trends similar to those found with reference oil were observed. Activities were the lowest in the hexadecane cultures. Growth on hexadecane/2-methylnaphthalene mixtures yielded activities that were intermediate between those on reference oil or hexadecane. Cell viability remained high after 10 days incubation. Emulsifying activity generally increased with time. In one instance, the activity decreased from 8 to 10 days (6% hexadecane). The reason for this decrease was not investigated, though one possibility might have been due to depolymerization of the emulsifying agent. Activities obtained on 2% hexadecane/2-methylnaphthalene mixture were generally higher than those at 6%. This trend contrasts with those found on reference oil or hexadecane, where 6% substrates induced higher emulsifying activities than 2%. *P. aeruginosa* UG1 cultures grown for 10 days in the absence of reference oil, hexadecane, or the hexadecane/2-methylnaphthalene mixture displayed negligible emulsifying activity (Tables 1 to 3, control values). Reference oil was not emulsified by components of LB broth in the absence of an inoculum. Therefore, the presence of hydrocarbon(s) was one of the conditions required for production of extracellular emulsifying agent. It is not presently known if emulsifying activity can be induced by other non-hydrocarbon substrates.

On fractional precipitation with cold ethanol, crude precipitates were obtained at each ethanol concentration used. All fractions collected contained some emulsifier activity (Table 4). The highest activity was exhibited by the material precipitated between 30 and 50% (v/v) ethanol. The precipitate in all the fractions was dark brown in colour. On diluting the precipitated material (from 0 to 1.0 mg dry weight/ml buffer), emulsifier activity declined linearly (Fig. 1).

The precise chemical nature of the emulsifier produced in this study is not known. Glycolipids containing the sugar rhamnose and β-hydroxycarboxylic acids have been isolated from *P. aeruginosa* [11]. Rhamnolipid was reported to display excellent

Table 4

Emulsifying activity of ethanol precipitated extracellular fractions produced by *P. aeruginosa* UG1

Ethanol Concentration % (v/v)	Emulsifying activity[a] (U/mg precipitate)
0–20	0.7
20–30	0.9
30–50	2.1

[a] All samples were corrected for blank; mean of two independent determinations.

surface activity and emulsifying ability as well as stimulating the growth of *P. aeruginosa* on hexadecane [11]. It also stimulated the growth of some *P. aeruginosa* strains on hexadecane, but not other bacterial species tested. It was, therefore, contended that one of the main functions of the extracellular rhamnolipid was to act as an emulsifying agent for the hydrocarbon [11].

Little is known about the genetic basis of emulsifier production. Plasmid-encoded growth on, and dispersion of crude oil, by *Acinetobacter calcoaceticus* RA57 have been reported by Rusansky et al. [23]. The *P. aeruginosa* strain investigated in this study did not contain any extrachromosomal plasmid DNA when subjected to alkaline-sodium dodecyl sulfate plasmid extraction [1] and agarose gel electrophoresis (data not shown). Some plasmid en-

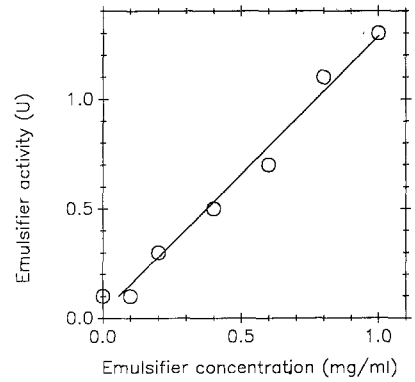

Fig. 1. Effect of precipitated emulsifier concentration on emulsifying activity. The correlation coefficient (0.99) and line of best fit ($Y = 1.32X - 0.02$) were calculated using a CMA statistic program (Educational Images, Lyons Falls, N.Y.) operated on an Apple II plus microcomputer.

coded functions can be highly unstable and lost due to plasmid curing at cell segregation. It may be advantageous to the *P. aeruginosa* strain to carry the genes required for emulsifier synthesis on the chromosome, where they are more stable. Since some surface-active molecules are basic components of cell surfaces, this is not an unusual finding.

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