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The mechanism of stabilization of actinomycete foams and the prevention of foaming under laboratory conditions

Linda L. Blackall[a] and Kevin C. Marshall[b]

[a]*Department of Microbiology, University of Queensland, St. Lucia, and* [b]*School of Microbiology, University of New South Wales, Kensington, Australia*

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SUMMARY

Cultures of *Nocardia amarae* give rise to cell-stabilized foams in a laboratory scale foaming apparatus. The organism produces a surfactant and the cells are very hydrophobic; factors which, in terms of froth flotation theory, are essential for foam production and transport of the cells from the aqueous to the bubble phase. The addition of montmorillonitic clay to the culture prior to foaming prevents foam stabilization. The results obtained suggest the formation of a salt-dependent, reversible, bacterium–montmorillonite complex which prevents transport of cells to the bubble phase.

INTRODUCTION

The formation of a stable biological foam on the surface of aeration tanks in activated sludge plants was first described in 1969 [3] and has been increasingly reported since then [11, 15, 18, 22]. Actinomycetes are the predominant organisms present; specifically *Nocardia amarae* [19] and *Rhodococcus rhodochrous* [22]. Of 52 activated sludge plants examined in south-east Queensland, continuous or intermittent foam problems were found at 48 sites [12]. *N. amarae* was the dominant organism in most foams, although another species of *Nocardia* was detected in some plants [12]. No single operation or design feature of the plants was found to be the cause of foaming.

According to froth flotation theory, both a frother surfactant and hydrophobic particles are required for generation of stable froths [20]. We have studied the growth of *N. amarae* in pure culture and have proposed a mechanism for the production by this organism of stable foams. Prevention of stable foam formation has been achieved by the addition of colloidal, hydrophilic clay particles.

Correspondence: Professor K. C. Marshall, School of Microbiology, P.O. Box 1, University of New South Wales, Kensington, N.S.W. 2033, Australia.

MATERIALS AND METHODS

Cultures and culture conditions

A field isolate of *N. amarae*, strain NM23, was used in all experiments. Cultures were grown on Yeast Glucose Agar (YG) containing (w/v): glucose 1%, yeast extract 1% and agar 1.5% (pH 7) prior to inoculation into 1 liter of Staniers mineral salts medium (MSM) [33] with 0.5% added carbon source in a 3 liter flask. For most experiments ammonium acetate was the carbon source, although growth on ethanol, glucose and trehalose was evaluated. Cultures were incubated at 28°C for 7 days on a gyratory shaker at 150 rpm. Washed cells were prepared by filtering the liquid culture through a 0.45 μm membrane filter, washing with 4 volume changes of either distilled water, MSM or phosphate-urea-magnesium (PUM) buffer [29], and finally resuspending in either water, MSM or PUM buffer, respectively. Dry weight determinations were made by filtering 20 ml aliquots of cultures through predried, preweighed 0.45 μm membrane filters and drying at 80°C until constant weight was attained.

Foaming, surface tension, hydrophobicity and clay addition

The surface tension of fluids was measured with an Analite Surface Tension Meter (Selby Scientific, Sydney, Australia) which employs the Wilhelmy Plate method. A foaming apparatus was constructed to allow compressed air to pass through a sintered glass disc and a liquid culture at 200 ml/min

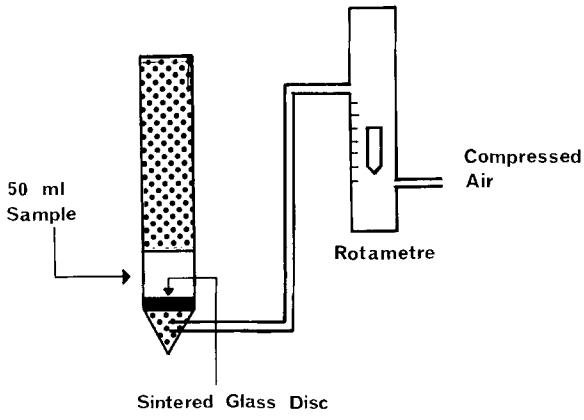

Fig. 1. Foam testing apparatus.

Table 1

Classification of foams generated in the laboratory foaming apparatus

Rating	Description
0	As for pure water: bubbles break surface but are unable to form or have no stability.
1	1.0–3.0 cm of foam with fragile, ill-formed bubbles. Insufficient stability to form films. Immediate collapse on cessation of aeration.
2	Intermittent films sufficiently stable to last for >5–10 s. Usually generated from a fragile foam structure of limited height. Films unstable on cessation of aeration.
2a	A slowly forming foam comprised of bubbles of about 1.0 cm diameter. The bubbles reach a height of about 2.0 cm then coalesce to form films across the cylinder which travel up the cylinder and are stable on cessation of aeration.
3	Substantial foaming (i.e. bubbles about 1.0 cm diameter) to 3–8 cm height. Infrequent or regular film formation, with both film and foam semi-stable on cessation of aeration.
4	Initially 8–15 cm of foam (about 1 cm diameter bubbles) with stable films being formed at regular intervals. Body of the foam and films stable for 3–5 min once aeration ceases.
5	Stable foam 5–10 cm in height in 2 min, after which collapse to 3–5 cm height. Foam is stable when aeration ceases. No films.
6	Stable foam 15–30 cm in height with no films. Bubble size about 0.5 cm during aeration and increases to 2.0–3.0 cm diameter in 3–5 min from the time aeration ceases.
7	Dense stable foam >30 cm in height over 2 min aeration. Bubble size about 0.3 cm during aeration and max. 1.0 cm diameter in 3–5 min after aeration ceases. Foam is sufficiently stable to show no change in height after a further 10–15 min.

(Fig. 1). The foam on the surface of a 50 ml liquid sample was assessed by an arbitrary rating system (Table 1). Cell surface hydrophobicity was assayed by the hydrocarbon affinity (HA) test using xylene and hexadecane [29]. *N. amarae* was inoculated into 100 ml of defined medium amended with 5 μCi of tritiated amino acids mixture (Amersham, U.K.) or tritiated sodium acetate (New England Nuclear, Boston, MA) and incubated. In the HA assay, 1.0 ml of cell suspension was taken from the aqueous

phase before and after assay, washed and added to 9 ml of Aquassure liquid scintillation fluid (New England Nuclear), the radioactivity was measured for 10 min in a Packard Tri-Carb Liquid Scintillation Spectrometer, and the results converted to disintegrations per minute (dpm).

Colloidal montmorillonite was prepared by suspending bentonite in distilled water, separating the coarse fraction by centrifugation at 12 000 × *g* for 20 min then concentrating the fine fraction by further centrifugation at 23 000 × *g* for 45 min [17]. A standard curve for montmorillonite concentration (μg/ml) versus absorbance at 245 nm [25] was used to assess the amount of montmorillonite added to the various preparations. Hydrophilic, colloidal clay was added at various concentrations to the *N. amarae* cultures and the effect upon surface tension, foaming and hydrocarbon affinity was monitored. Static tests were also carried out in 100 ml measuring cylinders. Whole cultures, distilled water-washed cells, MSM-washed cells and PUM buffer-washed cells in 50 ml amounts were amended with montmorillonite to a final concentration of 100 μg/ml and the appearance of the liquids was monitored over a period of time. The effect of the addition of 5 ml of culture filtrate, MSM or buffer to the water-washed cells was also assessed. The flocculation of 100 μg/ml montmorillonite by MSM, 0.1 MSM (MSM diluted 1 in 10) and PUM buffer was tested by allowing the suspensions to stand for 30 min followed by centrifugation at 1900 × *g* for 10 min. The concentration of clay remaining in suspension was determined spectrophotometrically at 245 nm [25].

Electron microscopy of bubbles

The technique for freezing bacteria on the surface of an intact bubble for examination in the electron microscope was developed by Sergei Kouprach, Biomedical Electron Microscope Unit, University of New South Wales. A copper specimen holder with a diameter of 15 mm was used to select bubbles from a shaken culture of *N. amarae* and then held in liquid nitrogen vapor until the thinnest part of the bubble began to freeze, whereupon the specimen and holder were plunged into liquid nitrogen and the frozen bubble placed on the rotating specimen holder of a Balzer BAF head, which had been precooled to –170°C. The chamber was evacuated and the knife cooled and placed above the specimen. The temperature of the specimen was raised from –170°C to –80°C, but the knife temperature was maintained at –196°C. Sublimation was allowed to take place for about 40 min to more clearly reveal the surface of the bacteria. The knife was then removed to allow shadowing and replica production in two steps: firstly, with Pt/C at 45°C, and, secondly, with carbon directly above the object. The specimen holder with the specimen and replica was then removed from the chamber, suspended in distilled water for several minutes, and transferred to bleach for several hours until the replica was clean. The replica was then washed in three changes of distilled water, placed on a 300 mesh copper grid, and observed in a Philips 300 electron microscope.

RESULTS

Seven-day-old cultures of *N. amarae* on the substrates tested yielded dry weights of 1.0–1.5 g/l, had surface tension values of 50–55 mN/m (compared to values for the uninoculated media of 66–67 mN/m, except for the ethanol medium at 55 mN/m) and produced stable foams in the foaming apparatus (Table 2). Continual washing of the culture resulted in a progressive increase in surface tension (from 50 to 70 mN/m), due to removal of the surfactant, and a dramatic reduction in foam stability (from a rating of 7 to 1 or zero) despite the presence of *N. amarae* cells. The culture filtrate (surface tension of 50–55 mN/m) gave good bubble production but the foam was unstable (Table 2). Addition of small volumes of culture filtrate to the washed *N. amarae* cells resulted in the formation of stable foams. The originally turbid culture became transparent on aeration as a result of the bacteria being transported to the bubble phase. In fact, the bubble lamellae were opaque. Electron microscope examination of carbon replicas of frozen (in situ) bubbles revealed a closely packed network of *N. amarae* cells with only minimal water layers inbetween (Fig.

Fig. 2. Electron micrograph of a carbon replica of a frozen bubble containing *N. amarae* cells. Note the closely packed network of cells (white arrow) and aqueous layers between the cells (black arrow). Bar = 1 µm.

2). Hydrocarbon affinity tests showed that virtually all of the *N. amarae* cells partitioned in the hydrocarbon phase (Table 2), indicating that the cells were very hydrophobic. The results obtained for foaming, surface tension and hydrocarbon affinity were similar irrespective of the carbon substrate used for growth of the organism (Table 2).

When montmorillonite (100 µg/ml) was added to *N. amarae* cultures prior to foaming, the foam formed had smaller bubbles than the control culture and lacked stability (Table 2). In addition, the cells did not enter the bubble phase and, upon cessation of aeration, the liquid phase remained turbid. The foam generated contained transparent bubble lamellae. An intermediate effect was observed when 50 µg/ml montmorillonite was added to the culture, and no additional response was elicited by more than 100 µg/ml.

When washed cells resuspended in distilled water, buffer or MSM were aerated, a large bubble foam resulted that developed into films extending across the area of the cylinder (rating 2a, Tables 1 and 2). The same effect occurred if montmorillonite was added to the distilled water-washed cells. The addition of 5 ml of either culture filtrate, MSM or PUM buffer to the distilled water-washed cell suspension plus montmorillonite resulted in an unstable foam (rating = 1). Addition of the same components to distilled water-washed cells in the absence of montmorillonite had no effect on foam formation. Both the buffer-washed cells and the MSM-washed cells supplemented with montmorillonite remained in the aqueous phase. In the static tests, whole cultures, MSM-washed cells and buffer-washed cells supplemented with 100 µg/ml montmorillonite flocculated after 10 min, and eventually the flocs settled to the bottom of the test cylinders. Distilled water-washed cells did not flocculate with clay alone, but flocculation ensued when 5 ml of either filtrate, MSM or buffer was added. Following flocculation, the surface tension of the liquid phase was the same as that in the absence of the clay (Table 2), suggesting that montmorillonite did not adsorb the microbially produced surfactant.

DISCUSSION

Adsorptive bubble separation techniques (adsubble processes) or froth flotation techniques are based upon differences in surface activity. Surface active material, which may be molecular, colloidal or macroparticulate in size, is selectively adsorbed or attached at the surfaces of bubbles rising through the liquid, and is thereby concentrated or separated [21]. These processes have been used to separate bacterial cells and spores by flotation [2,4,5,8,9,13,26,30,31,36].

The formation of laboratory-scale actinomycete foams conforms to the adsubble process theory. The *N. amarae* cells were hydrophobic and are the separated particles. *N. amarae* produced an uncharacterized surfactant required for foam generation, as evidenced by the lowered surface tension in both the whole cultures and the filtrate. The production of biosurfactants, especially by nocardioform actinomycetes, is well documented [1,7,10,11,16,23,24, 27,28,38], as is the property of deemulsification by *N. amarae* [6,34]. Both surfactant and cells are necessary for stable foam formation, since only unsta-

Table II

Foam production, surface tension and hydrocarbon affinity testing of *N. amarae*

Test conditions	Acetate-grown culture			Ethanol-grown culture			Glucose-grown culture			Trehalose-grown culture		
	foam rating[a]	surface tension[b]	HA[c]	foam rating	surface tension	HA	foam rating	surface tension	HA	foam rating	surface tension	HA
N. amarae culture	6–7	50–55	–	7	50	–	3–4	55	–	7	53	–
Cells washed once	1	56	–	–	–	–	–	–	–	–	–	–
Cells washed twice	0	61	–	–	–	–	–	–	–	–	–	–
Cells washed thrice	0	70	–	–	–	–	–	–	–	–	–	–
Filtrate	2	51	–	2–3	52	–	2	52	–	2	54	–
Complete culture + montmorillonite	2–3	50–55	–	5[d]	–	–	2	–	–	3	–	–
Distilled water-washed cells	2a	70	–	2a	75	–	2a	72	–	2a	71	–
Distilled water-washed cells + montmorillonite	2a	70	–	2a	–	–	2a	–	–	2a	–	–
Distilled water-washed cells + montmorillonite + salts	1	–	–	–	–	–	–	–	–	–	–	–
PUM buffer-washed cells	2a	–	0.9–1.6	2a	–	3.3	2a	–	5.0	2a	–	6.3
PUM buffer-washed cells + montmorillonite	1	–	2.8–4.3	1	–	13.3	1	–	7.1	1	–	9.5
MSM-washed cells	2a	–	–	–	–	–	–	–	–	–	–	–
MSM-washed cells + montmorillonite	1	–	–	–	–	–	–	–	–	–	–	–
Uninoculated medium	0	66	–	1	55	–	0	67	–	0	66	–
Uninoculated medium + montmorillonite	0	67	–	–	–	–	–	–	–	–	–	–

[a] See Table I.

[b] mN/m.

[c] Hydrocarbon affinity as % dpm remaining in the aqueous phase.

[d] The foam generated completely filled the cylinder but was unstable (note lower surface tension of uninoculated medium).

[e] –, not tested.

ble foams are formed in the absence of bacteria (culture filtrate) and no foams are formed in the absence of the surfactant (washed cells). We believe that the closely packed arrangement of cells within the bubble lamellae stabilizes the bubbles by ensuring that minimal distances are bridged by the water films (Fig. 2).

Because of the importance of cells in stabilizing foams, attempts were made to alter the nature of the cells to prevent their transport into the bubble phase. A variety of hydrophilic materials were added to cultures prior to aeration, but success was achieved only with the 2:1 expanding lattice clay, montmorillonite [14,37]. The lack of transport of *N. amarae* cells was not a result of adsorption of surfactant by the clay, since the surface tension of the liquid following flocculation of the clay and bacteria was the same as that in the absence of clay (Table 2). Similar results were obtained with bacteria grown on a variety of carbon sources, although the foam was more stable with ethanol-grown cells + montmorillonite because of the surfactant properties of ethanol [32].

The clay effect was concentration-dependent with the maximum effect at 100 μg/ml montmorillonite. Previous electrophoretic studies had shown that colloidal montmorillonite completely enveloped bacterial cells at a concentration of 100 μg/ml [17,25]. Such an envelope would result in an apparently hydrophilic, rather than hydrophobic, outer surface. This bacterium–clay complex must be a loose association as the hydrocarbon affinity test of the bacteria in the presence of montmorillonite showed that most of the bacteria still partitioned into the hydrocarbon phase (Table 2). A stable foam was formed when *N. amarae* cells washed in distilled water were aerated in the presence of montmorillonite. The addition of either MSM, PUM buffer or filtrate is required for the foam destabilization effect to be elicited. PUM buffer, but not 0.1 M MSM or MSM, flocculated a suspension of 100 μg/ml clay. However, all of these salt solutions produced a visual flocculation effect in a suspension of 100 μg/ml clay in distilled water-washed cells.

We postulate that a salt-dependent, reversible bacterium–montmorillonite complex [35] is rapidly formed which confers hydrophilicity to the otherwise hydrophobic actinomycetes. This property prevents cells from entering and stabilizing the foam phase in an appropriately aerated pure culture. The practical implications of this phenomenon could be widespread in the wastewater field, although evaluation of the addition of clay to foaming activated sludge plants needs to be carried out to confirm the efficacy of the treatment. More fundamental investigations into the actual mechanism of foam prevention by the clay need to be initiated.

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