Journal of Industrial Microbiology & Biotechnology (2002) 28, 264–267

www.nature.com/jim

Cell surface hydrophobicity and mycolic acid composition of *Rhodococcus* strains isolated from activated sludge foam

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The bacteria causing foaming in activated sludge plants are considered to be hydrophobic, and their hydrophobicity is assumed to be a crucial factor in their foam-forming ability. This study showed no consistent relationship between cell surface hydrophobicity (CSH), as determined by microbial adherence to hydrocarbons, of three *Rhodococcus* spp. isolated from activated sludge foam and their ability to produce a stable foam. There also appeared to be no correlation between the mycolic acid composition of these strains, in terms of chain length or degree of unsaturation, and either CSH or foaming ability. Zeolite and bentonite successfully prevented foaming by a *Rhodococcus* sp. in pure culture, which suggests that cell surface charge may also play a role in foam stabilisation.
Journal of Industrial Microbiology & Biotechnology (2002) **28,** 264–267 DOI: 10.1038/sj/jim/7000241

Keywords: activated sludge foam; mycolic acids; cell surface hydrophobicity; *Rhodococcus*

Introduction

Activated sludge plants all over the world suffer continually or sporadically from the serious operational problem of foaming, where a persistent brown viscous foam develops on the surface of the aeration tanks, leading to many problems [15]. Foaming is a microbiological problem. Under the microscope, branched and unbranched filamentous bacteria, as well as unicells [15], including some possible pathogenic bacteria [20], are observed in large numbers. The two most commonly observed groups of organisms in foams include the unbranched filament, *Microthrix parvicella*, and members of the Gram-positive mycolata, branched bacteria containing mycolic acids in their cell walls [15]. Although *Nocardia amarae* (now called *Gordonia amarae*) was once thought to be the most common microbe responsible for foaming, it is now clear that the taxonomic diversity of foaming mycolata is substantial [15]. The mycolata include members of several different genera, which look very similar under the microscope, all possessing short branching filaments [18].

Little is understood about the factors that might affect foam formation in activated sludge plants [3,15] or the actual mechanisms involved. Pujol *et al* [13] suggested that a combination of filamentous organisms, air bubbles and "preferential substrates" is necessary for foam formation. One likely hypothesis is that foam forms in the presence of air bubbles and surfactants and is subsequently stabilised by the flotation of hydrophobic particles, in this case bacterial cells [15]. The presence of surfactants assists by allowing the walls of the air bubbles to remain elastic and the hydrophobic parts help prevent liquid draining from the bubble walls [11]. Therefore, the involvement of bacterial cell surface hydrophobicity (CSH) in foam formation is a crucial element of the flotation theory [2].

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Received 12 December 2000; accepted 18 December 2001

It is assumed that the mycolata are important foaming bacteria because they contain long-chain branched mycolic acids in their cell walls which impart CSH [2]. However, previous studies have generally concentrated on relating the carbon chain length of the mycolic acids to CSH and the little data available suggest that cells with longer-chain mycolic acids are more hydrophobic than those containing acids with shorter chains [1]. This study examines relationships among the CSH of isolates of three *Rhodococcus* spp. obtained from activated sludge foams, their mycolic acid compositions and their abilities to form foam.

Materials and methods

Isolates

The three *Rhodococcus* isolates used in this study and their growth conditions have been described [21]. All were from activated sludge foams and were isolated as Gram-positive branched filaments that were considered to be responsible for the foam when samples were taken. The choice of growth media used for cell surface studies was based on previous experience with activated sludge isolates [17]. The carbon sources were selected to provide either a hydrophobic (Tween) or hydrophilic (glucose) substrate for these cell surface and foaming studies.

Mycolic acid analysis

Mycolic acids were analysed using GC-MS of their methyl ester trimethylsilylether derivatives with quantification of fragment ions by selective ion monitoring [21,19]. The analytical error for individual mycolic acid values was estimated to be no more than 2% between duplicate samples. This method provides detailed structural information which reveals the true extent of chemical diversity of these fatty acids in the bacteria under investigation [21,19].

Determination of CSH

CSH was quantified using the microbial adherence to hydrocarbon (MATH) assay described by Rosenberg *et al* [14], with *n*-hexadecane as the solvent. Percentage hydrophobicities were determined from $(A_{540\ nm}\ \text{initial} - A_{540\ nm}\ \text{final})/A_{540\ nm}\ \text{initial} \times 100$) [7]. This method was preferred to others, such as the hydrophobic interaction chromatography technique where the filamentous morphology of the rhodococci may result in physical entrapment of the cells rather than their adherence to the column material from hydrophobic interactions.

Assessment of foaming ability of strains

A foaming apparatus similar to that described by Blackall and Marshall [2] was constructed, consisting of a 250-ml measuring cylinder with a sintered glass disc fitted to its base, which was connected to a rotameter. After a 20-ml volume of broth culture ($A_{540\ nm}$ adjusted to approximately 1.0.) was added to the cylinder, air at a rate of 100 ml min^{-1} was bubbled in. Foam generation was assessed according to the criteria of Blackall and Marshall [2] in terms of its volume, stability and bubble size and rated from 0 to 7. Zero was equivalent to pure water with no bubbles formed and 7 was a dense stable foam with 0.3-cm bubbles during aeration and 1.0-cm bubbles that were stable for more than 5 min after aeration had ceased. The percentage of cells removed from the bulk liquid was also measured to assess the partitioning of cells into the foam by measuring its absorbance at 540 nm after 1 min of foaming.

Chemical control of foam formation

Reagents assessed as antifoam agents (see Results section and Figure 1) were used as 10 mg ml^{-1} solutions in deionised water. The Zetag and Magnafloc suspensions were prepared according to the manufacturer's instructions (Ciba Specialty Chemicals, Wyong, Australia). These were added to cultures to a final concentration of 500 μg ml^{-1}, and their effects on the foaming abilities of treated cultures were determined [2].

Two clays were also tested as antifoam agents. The bentonite (Sigma Aldrich, Sydney, Australia) (i.e., aluminium silicate arranged in sheets) carries a negative charge on the face of the sheets, while the edges are positively charged. Muloorina (a tubular aluminium silicate) is nonspecifically charged. Suspensions of bentonite were prepared according to the method of Lahav [10], while muloorina, a gift from P. Slade (CSIRO, Adelaide, Australia), was prepared in suspension according to his instructions.

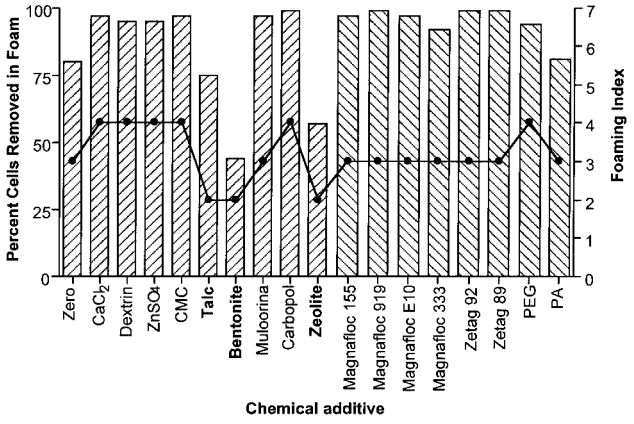

Figure 1 Manipulation of the foaming ability of 14-day *Rhodococcus rhodochrous* 11R cells grown in 1% glucose at 25°C. The percent of cells removed from the bulk liquid was determined as decrease in the optical density (540 nm ▨) and the foaming index (●), according to Table 1. PEG, polyethyleneglycol; PA, polyacrylamide; CMC, carboxymethylcellulose.

Results

Relationship between mycolic acid composition and CSH of Rhodococcus isolates

The three *Rhodococcus* isolates responded differently to changes in growth temperature and carbon source, in terms of both their mycolic acid compositions and measured CSH (Table 1). No clear correlation was observed between these parameters. For example, cells of *Rhodococcus* 11R grown at 17°C with Tween 80 contained no mycolic acids, yet they were very hydrophobic. No apparent relationship was noted between the measured CSH and either levels of saturation/unsaturation or carbon chain lengths of the mycolic acids in any of the three *Rhodococcus* isolates. Thus, when grown with glucose at 32°C, strain D5 had 93% saturated mycolic acids and strain 11R was 55% saturated (Table 1), yet their CSH were both similar. Strain 11R grown on glucose at 17°C had a lower CSH than strain A7 under the same conditions (Table 1), but the former had a much higher proportion of longer-chain mycolic acids (data not given).

Relationship among CSH, mycolic acid composition and foaming abilities of Rhodococcus isolates

As with mycolic acids and CSH, no consistent relationship was detected between the foaming abilities of cells under the conditions used here and their CSH. In some cases, high CSH appeared to be associated with production of a stable foam (e.g., *Rhodococcus* strain 11R grown with Tween 80 at 17°C), but more commonly it did not. Cells with lower measured CSH produced more stable foams, e.g., strain D5 grown on Tween 80 at 17 and 32°C (Table 1). Similarly, the data did not support any consistent relationship between mycolic acid chain length or levels of their saturation and unsaturation, and the foaming abilities of cells. In fact, the opposite to what might have been expected from earlier published data on the relationship between foaming ability and mycolic acid composition was often noticed [1]. Thus, strain D5 grown at 25°C with glucose had shorter-chain mycolic acids than strain 11R grown under the same conditions, yet both the foam stability and foam-associated cell numbers were much higher in strain D5 than in strain 11R (Table 1). These trends are even more striking with some of the other members of the mycolata examined in this laboratory, e.g., *G. amarae* (unpublished data). Although there was usually a reduction in the surface tension of the medium, consistent with production of surfactants, this decrease did not always correspond to changes in either the foaming abilities of the cells or foam stability (Table 1).

Other hydrophobic cell surface components

The results above suggest that mycolic acids may not be the only factor contributing to CSH or the foaming ability of these bacteria. For example, *Rhodococcus* strain 11R was extremely hydrophobic and produced a stable foam under conditions where mycolic acids levels were near the detection limit of the analytical procedures used here (Table 1). Both *Rhodococcus* strains A7 and D5 formed capsules, and these capsulated forms always had a higher CSH than the noncapsulated strains, unlike the strains used by Sunairi *et al*

Table 1 CSH, foaming ability and mycolic acid composition of three *Rhodococcus* isolates, grown on 1% glucose or Tween 80

Isolate	Growth temperature (°C)	Carbon source[a]	% CSH	Foaming ability[b]		Carbon chain length range	Mycolic acid composition percent of total (degree of saturation)			
				1	2		Saturated	Monounsaturated	Diunsaturated	Triunsaturated
11R	17	G	64±6	84	2	32–44	16.5	58.0	25.5	0.0
		T	85±2	40	5–6					
	25	G	44±11	79	3	32–46	44.1	42.0	13.9	0.0
		T	60±6	26	3	32–42	15.7	77.3	7.0	0.0
	32	G	73±10	96	3	32–44	54.8	29.8	15.4	0.0
		T	67±12	12	3	32–41	2.6	86.8	10.6	0.0
A7	17	G	71±2	72	3	32–44	47.2	42.4	9.6	0.8
		T	67±3	18	5	32–43	28.0	34.3	33.1	4.6
	25	G	59±8	86	3	32–40	55.1	42.8	2.1	0.0
		T	70±3	21	2	32–43	31.7	44.5	23.8	0.0
	32	G	59±10	48	2	32–43	75.1	21.6	1.3	2.0
		T	57±9	21	2	32–44	62.1	31.2	2.8	3.9
D5	17	G	60±3	88	3	31–42	72.9	24.7	2.4	0.0
		T	69±2	23	7	32–45	37.4	43.5	8.8	10.3
	25	G	63±6	92	5	32–42	73.4	26.6	0.0	0.0
		T	54±5	24	3	32–45	60.5	34.1	2.5	2.9
	32	G	74±4	69	2–3	32–38	93.2	6.8	0.0	0.0
		T	67±4	28	7	32–45	56.4	36.4	7.2	0.0

Stationary phase cells were used in the analyses.
[a]G, glucose; T, Tween.
[b]One percent of cells removed from bulk liquid during foaming test; 2, 0-7 Foam Index adopted from Ref. [2].

[22]. However, these noncapsulated strains did not necessarily produce a less stable foam.

Manipulation of foaming abilities of Rhodococcus strain 11R

Rhodococcus strain 11R was chosen for these experiments since with 1% glucose as carbon source, it produced a substantial stable foam under the test conditions used. Of the many compounds examined as antifoam agents, only zeolite and bentonite clays prevented foam formation (Figure 1). A slight reduction in foaming index was observed with talc, although more cells moved into the foam layer than moved after exposure to the clays (Figure 1). In other trials, the chemicals tested either had little effect on the foaming abilities of this strain or increased both the stability of the foams and the levels of cell biomass carried up into the foam from the bulk liquid during the foaming test (Figure 1). However, in neither bentonite nor zeolite was there any change in the measured CSH of the culture after their addition (data not shown). Such data would further question a role for CSH (as measured by the MATH assay) in determining the foaming ability of these organisms. Similar data trends have also been observed with a *G. amarae* strain isolated from foams (data not shown).

Discussion

Although an earlier survey [19] linked foaming in activated sludge plants with changes in biomass CSH by the MATH assay, similar studies failed to convincingly support these observations [9]. Kahn *et al* [9] proposed that the higher the measured CSH (they also used the MATH assay) of the sludge, the more likely it was to form foam. Although it is not possible to mimic the fluctuating conditions that occur in full-scale activated sludge system in the pure cultures of *Rhodococcus* foam isolates, no convincing evidence was obtained relating CSH and foaming ability. Neither was there any consistent relationship between CSH of cells and the chain lengths of their mycolic acids, even though both were altered in response to changing culture conditions. These inconsistent findings between this pure culture study and other sludge studies [3,5,9,19,20] may result partly from the methods that have been used to determine CSH, since all have limitations [4]. Even so, the MATH assay has been used as the method of choice in most similar studies attempting to relate activated sludge hydrophobicity and CSH of foaming bacterial isolates [2,5].

If a cell must be hydrophobic to participate in foam formation, then modifying the cell surface so that it becomes hydrophilic may help prevent foam formation. Blackall and Marshall [2] suppressed foam formation by addition of colloidal bentonite to *G. amarae*, suggesting that the clay was masking cell surface hydrophobic groups, thus creating a hydrophilic particle. The CSH of other microbes has also been manipulated by addition of a range of chemicals. For example, adsorption of polycationic polymers to negatively charged surface carboxyl groups rendered cells more hydrophobic [23]. The addition of antifoams to activated sludge as a foam control strategy is still used [8] and some success has been reported with these antifoam chemicals against *G. amarae* foams [16]. Most of the polyelectrolytes, cations and clays tested here were unsuccessful in controlling foam formation in *Rhodococcus* strain 11R, similar to the results reported for *G. amarae* [2].

Blackall and Marshall [2] demonstrated that *G. amarae* cells in the presence of bentonite still partitioned into the organic phase during the MATH assay, but did not form a stable foam. It is thought likely that these clays adsorb to negative charges on the bacterial cell surface *via* positive charges on their ends, thus coating the cell. Parallel SEM and TEM studies with *G. amarae* (unpublished) showed that clay particles in fact align perpendicularly around the cells *via* their edges. The failure here of the nonspecifically charged muloorina clay particles to either influence foam formation or attach to the cell surface of *Rhodococcus* strain 11R supports a view that the mechanisms for clay binding are surface charge-related [2].

Zeolites, insoluble inorganic ion exchangers [24], also reduced foaming, but appeared to work differently by causing the bacterial cells to clump, and so prevented them from adhering to floating

bubbles, possibly by modifying their surface charge. Talc has also been reported to control bulking in activated sludge [6]. It appeared to have some preventative effect here with the *Rhodococcus* strain 11R (Figure 1), and addition of talc substantially reduced foaming in *G. amarae* (unpublished). Again its mechanism of action is not understood, but claims that talc is hydrophobic are not consistent with these data.

Addition of solids to activated sludge for foam control is undesirable since these create extra solids for eventual disposal. However, bentonite clay is effective at very low concentrations and has been used successfully for controlling foam in a full-scale plant (R.J. Seviour, unpublished). It may be feasible to remove foam from the reactor, and then add the clay to it to destabilise it. As foaming is seen by many engineers as the one of the last major unsolved problems in activated sludge treatment processes, such a control strategy may still be better at this stage than attempts at foam prevention. Understanding of the microbial ecology of foam and mechanisms for its formation is still inadequate for this to be a reliable control of foaming in activated sludge systems [15].

The data in this study raise doubts about our current understanding of mechanisms for foam formation. The evidence shows cell surfaces of foam-forming bacteria, such as the rhodococci change in terms of their wall chemistry, and their physical properties, such as surface charge, after adsorption of chemicals. It may be appropriate to modify the emphasis placed so far on cell surface hydrophobic components and consider foaming from a wider perspective.

A common experience is that foaming incidents occur too quickly to be explained in terms of changes in bacterial surface chemistry. Other characteristics of the bulk liquid within the aeration tank also change very rapidly. Interactions between cell surfaces and the bulk liquid are likely to be important, and may provide another partial explanation for foaming. These interactions depend on both the nature of the cell surface and the liquid surrounding it. For instance, a bacterium in a medium where the electrostatic interaction is equal to or close to zero (zeta potential=0) will exhibit its highest CSH [4]. Thus, if zeta potentials (and hence CSH) change with influent properties, or following a pH change from a process like nitrification or denitrification, sudden foam formation may result. Addition of chemicals like ferric chloride or aluminium compounds, often used to improve settling or enhance phosphorous removal, will also change the ionic properties of the bulk liquid, in ways consistent with this hypothesis.

Most influents contain large amounts of hydrophobic substrates such as fats and oils. Some studies have shown that hydrophobic cells like *Rhodococcus* can attach to these [12] and possibly gain a competitive advantage over hydrophilic cells [15,17]. By a similar mechanism, some foaming bacteria appear to attach quite firmly to hydrophobic substrates and under aeration are carried up out of the bulk liquid into the foam layer. This may also partly explain their involvement in foam formation and account for the sudden episodic outbreaks of foaming. If so, the control of activated sludge foaming, caused in several different ways, will be difficult.

Acknowledgements

Financial support was provided by the Victorian Education Foundation and Sydney Water.

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