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Isolation of bacteria producing siderophores under alkaline conditions

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Summary. The isolation of bacteria producing siderophores under alkaline conditions is reported. Enrichment cultures initiated with samples from a number of alkaline environmental sources yielded 80 isolates. From this group selections were made on the basis of growth at high pH and the gallium-binding capacity of the siderophores. It was found that some isolates grew well and high concentrations of siderophore were detected whereas others grew well in the presence of much lower concentrations of siderophore. The effect of iron, gallium and aluminium on growth and siderophore production in batch culture was investigated for six isolates. The presence of iron greatly decreased the siderophore concentration in these cultures, whereas the response to added gallium or aluminium was dependent upon the isolate.

Introduction

During the early 1980s gallium arsenide emerged as an alternative material to silicon in electronic devices. In 1983 the estimated consumption of gallium in the electronics industry was 3.3×10^3 kg with an expected annual increase in demand of 5–10%, until the year 2000 (Wardell and Davidson 1987). Indeed Schoenung and Clarke (1987) made a market prediction demand of 7.5×10^3 kg by 1990.

Although gallium is found in many minerals it is not naturally concentrated. It is extracted by electrochemical means from Bayer liquor waste resulting from aluminium production. Due to the low concentration of gallium in Bayer liquor relative to aluminium and sodium (Ga, 100–200 mg/l: Al_2O_3, 80–110 g/l; Na_2O, 150–240 g/l; Pesic and Zhou 1988), a very specific means of extraction is required. Acid leaching and Kelex 100 resin have been investigated as alternative methods (Pesic and Zhou 1988; Wardell and Davison 1987). It proved possible to leach gallium from a zinc-processing residue with sulphuric acid, but a high percentage of the zinc and cadmium remaining in the residue was extracted simultaneously (Wardell and Davidson 1987). Kelex 100 offers higher selectivity, but the kinetics of the extraction are very slow and whereas sodium up to a certain concentration had a positive effect, aluminium had a negative effect on the solvent extraction of gallium (Pesic and Zhou 1988). Bayer liquor is extremely alkaline ($pH \geq 13$) so any method of extraction operative at high pH would be economically attractive.

The aim of the work reported here was to investigate the potential of microorganisms, their products or chemically derived analogues thereof in the extraction of gallium from materials such as Bayer liquor. To this end we have exploited the production by microorganisms of low relative molecular mass metal chelators (siderophores). Bacteria and fungi excrete siderophores in order to facilitate the acquisition of Fe(III) from the environment. Although it is abundant, much of the iron is in an insoluble form at physiological pH. Although siderophores are generally considered ferric-specific, they have been shown to bind other trivalent metals, notably gallium and aluminium (Emery 1971; Llinás et al. 1970); metals other than iron are usually less strongly bound. Gallium is the only metal known to compete with iron for siderophore binding. Indeed if the Fe(III) of ferrichrome is chemically replaced with Ga(III) the ferrichrome uptake system of the smut fungus (*Ustilago sphaerogena*) is tricked and the gallium derivative is taken up at the same rate as the ferri ferrichrome (Emery and Hoffer 1980).

This paper reports the isolation and selection of organisms capable of growth and producing gallium-binding siderophores under alkaline conditions, as a first step in assessing the potential of a siderophore-mediated extraction procedure for gallium.

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Materials and methods

Culture media. Three media were used throughout these experiments. For the initial enrichments the defined medium of Dunn and Bull (1983) was used with TRIS buffer at 40 mM and at 100 mM or with $CaCO_3$ (50 g/l) added as additional buffering capacity (pH 8.7). This medium was used with no added iron (Fe concentration, 0.2 μM), hereafter referred to as "low" Fe, and with Fe added at 10 μM, hereafter referred to as "high" Fe. The defined medium was used with 100 mM TRIS for axenic cultures. For higher pH enrichments a complex medium described by Horikoshi and Akiba (1982) was used, initially with borate buffer (50 mM, pH 10.5) and in later experiments with glycine buffer (20 mM, pH 10.6) in place of sodium carbonate. A third semi-defined medium was formulated as follows: glucose, 10 g/l; yeast extract, 2.5 g/l; K_2HPO_4, 1 g/l; and added after sterilisation NaOH (10 M stock solution) 3 ml/l; $MgSO_4$, 0.15 g/l; trace mineral solution minus iron (Dunn and Bull 1983), 1 ml/l; glycine buffer (200 mM, pH 10.6), 100 ml/l. The semi-defined and complex media were routinely treated with hydroxyquinoline prior to autoclaving to reduce the iron concentration (Waring and Werkman 1942). Iron concentrations after treatment were approximately 150 μg/l (2.7 μM) in the complex medium and approximately 40 μg/l (0.7 μM) in the semi-defined medium. The defined medium was not treated since the iron concentration was already low (as mentioned above). The iron content of all media was determined by atomic absorption spectroscopy. All cultures were incubated at 25° C.

Samples. Environmental samples were collected from four geographical areas (UK, Ireland, USA and Indonesia). From most locations multiple samples were available from different alkaline sites (Table 1).

Enrichment and isolation. Conical flasks (300 ml) containing 40 ml defined medium were inoculated with 1 g (fresh weight) sample from the various sites listed in Table 1. Culture density and pH were monitored as growth proceeded. A 10% (v/v) inoculum was transferred at regular intervals. At each transfer, dilution series of the cultures were plated on defined and blue agars (see below). Isolates were selected from both types of agar and checked for growth on the other. Further enrichments were made at higher pH using the complex medium. Cultures from this latter series were plated on complex medium agar.

In growth studies culture density was monitored at 540 nm using a Perkin Elmer (Beaconsfield, UK) Lambda 1 UV/Vis spectrophotometer. Culture pH was monitored using a probe attached to a Phillips (Eindhoven, The Netherlands) PW 9418 pH meter.

Isolates were purified by successive transfer on the requisite agar and checked by microscopy.

Siderophore assays. The presence of siderophores was monitored by the universal siderophore assay of Schwyn and Neilands (1987). This colorimetric assay, employing chrome azurol S (CAS) is based upon the ability of siderophores to bind iron and does not distinguish chemical structure. The Schwyn and Neilands assay was modified by the inclusion of gallium or aluminium at the equivalent concentration in place of iron to assay the ability of siderophores to bind these metals. The CAS-shuttle solution was used for all three metal assays by the addition of sulphosalicylic acid, as described by Schwyn and Neilands (1987). The commercially available siderophore, Desferal (Ciba Geigy, Horsham, West Sussex, UK), was used as a standard to relate siderophore concentrations in culture supernatants (after centrifugation) to "Desferal equivalents" in order to enable comparisons between cultures to be made. Samples from cultures were compared to uninoculated media controls and Desferal standards were prepared in the requisite buffers. The dye-detergent-metal complex (CAS assay solution) can also be incorporated into agar. Microbial colonies excreting siderophores are surrounded by orange halos. These "blue agar" plates (Schwyn and Neilands 1987) were used in the isolation procedure.

Table 1. Environmental samples, pH, and number of isolates selected from each of two different media

Sources and sample number	pH	Number of isolates	
		Enrichment medium	
		Defined	Complex
Chalk soil (Kent, UK)			
1	8.4		
2	8.0	49	5
3	7.9		
Alkaline soils (Indonesia)			
1	9.0		
2	7.8		
3	8.3	NE	5
4	9.2		
5	8.0		
6	8.0		
Cement works waste (Kent, UK)			
1	9.5		
2	8.2		
3	9.6		
4	8.6	NE	15
5	9.1		
6	8.7		
7	8.6		
8	9.9		
Loch water sediment (County Clare, Eire)			
1	9.4	NE	4
2	7.3		
Dry soda lake soil (Oregon, USA)	10.0	NE	2

NE = no enrichment

For the initial characterisation of siderophores produced by isolates, two assays that distinguish chemical structure were used. One (Arnow 1937) detects catecholate-type siderophores and was used with 2, 3-dihydroxybenzoic acid as a standard. The absorbance of the assay solution was determined at a wavelength of 510 nm. The presence of hydroxamate-type siderophores was determined by an assay described by Atkin et al. (1970) using Desferal as the standard. We found that maximum absorbance occurred at 475 nm, rather than 495 nm as cited by Atkin et al. (1970) so the shorter wavelength was used.

Glassware. All glassware used for culturing organisms and manipulating and storing samples was acid washed (10% HNO_3) for at least 48 h before being rinsed three times with deionised distilled water.

Results

Modification to the siderophore assay

The inclusion of gallium or aluminium in the siderophore assay resulted in slightly different coloured assay solutions, gallium being somewhat browner and aluminium forming a more intense blue coloration than the iron assay solution. Scanning spectra were measured on

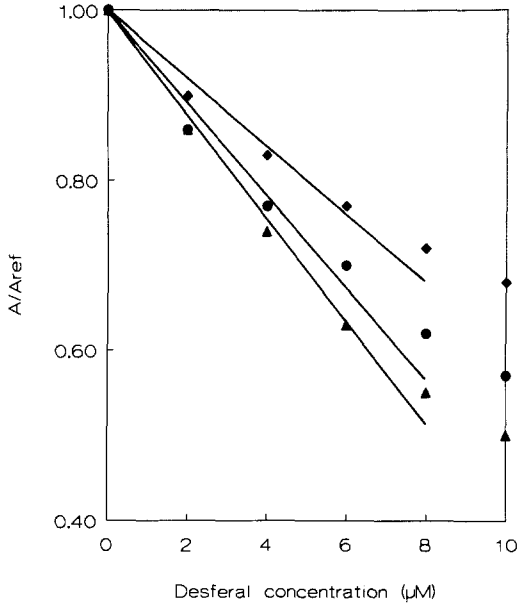

Fig. 1. Standard curves for siderophore assays. Desferal standards were prepared in glycine buffer and assayed with CAS solutions: ▲, CAS-Fe; ■, CAS-Ga; ▲, CAS-Al. A/A_{ref}, absorbance of the sample as a proportion of the absorbance of the assay solution with no siderophore present

the blank samples to determine absorbance maxima for the assays. The result for iron at 630 nm was in agreement with the published results of Schwyn and Neilands (1987): aluminium showed maximum absorbance at the same wavelength, but for gallium the maximum was at 640 nm. Desferal standards over the range 0–10 μM were used: a linear response was seen with all three assays over the range 0–8 μM (Fig. 1), but there was some departure from linearity with the 10 μM standard. There was good reproducibility of results over a period of 5 days with standard deviations of less than 10% and usually less than 5%. However, standard and assay solutions deteriorated with storage and regular preparation was required.

Enrichments and selection

The sampling sites, their pH and the number of isolations made from each, on the two media used are listed in Table 1. The two enrichment series yielded 80 bacterial isolates. The first stage of the selection procedure involved growing the isolates axenically in liquid culture and quantifying siderophore production in relation to Desferal standards. Isolates originally selected in a complex medium were transferred to the semi-defined medium in order to reduce the iron concentration of the medium. Selection was made on the basis of two criteria, culture density and siderophore production (e.g., high culture density and low siderophore production, low culture density and high siderophore production) to allow the possibility of high and low affinity systems to be selected. Isolates were examined for their morphology on plates and by microscopy to ensure that a diversity of systems was chosen. Through this process

Table 2. Growth and siderophore concentration for the 17 isolates selected

Isolate	Maximum culture density (OD_{540})	Siderophore[a] concentration (μM)	
		Fe assay	Ga assay
Defined medium[b]			
15	1.8	48	102
20	0.87	16	25
22	0.95	12	59
26	0.85	18	48
36	0.73	0	0
37	0.86	21	114
39	1.5	10	13
42	2.1	12	15
47	1.2	45	122
Semi-defined medium[b]			
E	0.65	0	0
E1	2.3	125	72
E2	1.4	83	48
E3.2	2.6	2.5	0
H	4.3	2.7	1.8
O	10.4	52	20
V	1.1	13	0
W2	2.8	215	179

OD_{540} = optical density at 540 nm

[a] The siderophore assay of Schwyn and Neilands (1987), which measures siderophore concentration in relation to the ability of the chelator to remove iron from an Fe(III)-dye-detergent complex, was modified by replacing the Fe(III) with Ga(III). The siderophore concentrations reported here are related to Desferal equivalents for the two assays

[b] Enrichments were carried out using two media. The defined medium has a pH of 8.7 whereas the semi-defined medium has an initial pH of 10.6. Organisms were cultured on the medium used for their enrichment and isolation. The media did not contain added gallium

17 isolates were selected (Table 2). At this stage the borate buffer used in both the complex and the semi-defined media was replaced with glycine buffer in order to facilitate metal analysis at a later stage.

In the second stage of the selection procedure the 17 isolates were tested for growth/survival in the presence of gallium and for the ability of their siderophores to bind gallium (Table 2). Gallium was added to liquid media at 10 μM, greater than a tenfold excess to iron, to allow for any toxicity problems associated with gallium-siderophore binding to be detected. At this concentration gallium had little apparent effect on the final culture densities reached by the isolates (results not shown). The isolates showed a variety of metal-binding abilities (Table 2). With isolates enriched and grown on defined medium, the Desferal equivalent concentration was generally higher for the gallium assay (Table 2), in some instances considerably higher (e.g., isolates 22 and 37). For those isolates enriched and grown on semi-defined medium, the Desferal equivalent concentrations for the gallium assay were always lower than those for the Fe assay. Although these results enable

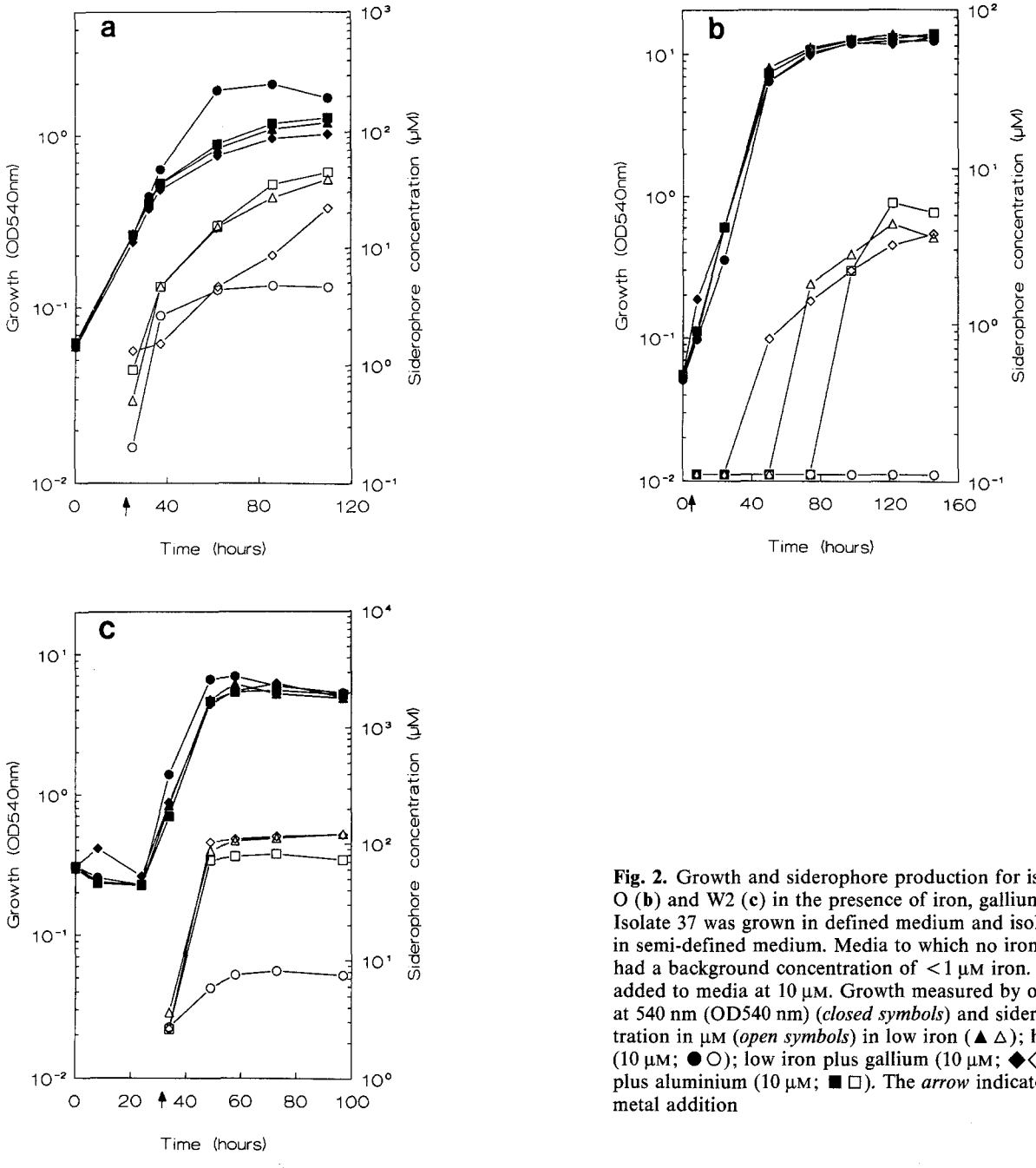

Fig. 2. Growth and siderophore production for isolates 37 (**a**), O (**b**) and W2 (**c**) in the presence of iron, gallium or aluminium. Isolate 37 was grown in defined medium and isolates O and W2 in semi-defined medium. Media to which no iron was added had a background concentration of <1 μM iron. Metals were added to media at 10 μM. Growth measured by optical density at 540 nm (OD540 nm) (*closed symbols*) and siderophore concentration in μM (*open symbols*) in low iron (▲ △); high iron (10 μM; ● ○); low iron plus gallium (10 μM; ◆◇); low iron plus aluminium (10 μM; ■ □). The *arrow* indicates the time of metal addition

comparisons of isolates to be made, the assay involves competition for binding. Because the binding coefficients of the assay complexes involving iron and gallium are not known, the Desferal equivalent concentrations are not directly comparable.

From these results a selection of six isolates was made for more detailed study. Isolates 15 and 37 were chosen because the capacity of their siderophores to bind gallium was apparently considerably higher than their binding of iron. Isolate H was chosen for its high biomass production but low siderophore production for both gallium and iron. Isolate O produced a very high culture density in the semi-defined medium and it attained intermediate siderophore production. Finally, isolates E1 and W2 produced good growth and very high siderophore titres with gallium and iron.

Time-course experiments were made with these six isolates during which siderophore production in relation to growth in batch culture was followed. Aluminium was introduced into the system since it is a major constituent of Bayer liquor and some siderophores are known to bind aluminium (Emery and Hoffer 1980). The presence of gallium or aluminium in the media at 10 μM had little apparent effect on the specific growth rate or the final culture density (typical data are shown in Fig. 2). However the presence of added Fe (10 μM) extended the exponential phase and thereby increased the final culture densities of isolates 37 (Fig. 2a) and 15 (not shown). Siderophore production apparently commenced at different stages of the growth cycle with different isolates. For isolates 37 and W2, siderophore production commenced during mid- to late-exponential

Table 3. Siderophore production by isolates growing in the presence of added iron, gallium and aluminium at alkaline pH

Isolate	Siderophore[a] concentration (μM)			
	Low Fe[b]	Plus Fe[c]	Plus Ga[c]	Plus Al[c]
15	51	5	41	52
37	42	5	22	49
El	55	4	20	60
H	<1	4	3	4
O	4	0	4	5
W2	106	5	116	72

Isolates 15 and 37 were grown in defined medium, the other four isolates in semi-defined medium

[a] Siderophores were measured using the CAS (Fe) assay and the response related to Desferal standards. Siderophore concentrations relate to the maximum culture density

[b] Low Fe media has no added iron; the background of iron was <1 μM

[c] Iron, gallium and aluminium were added at 10 μM

phase (Figs. 2a and c) but whereas isolate W2 apparently ceased siderophore production on entering the stationary phase (Fig. 2c), siderophore production of isolate 37 continued when the growth rate slowed (Fig. 2a). With isolate O siderophore production did not start until the culture was in the stationary phase (Fig. 2b).

By use of the siderophore assays with each of the three metals of interest, it was determined that all the siderophores produced by the isolates were capable of binding Fe(III), Ga(III) and Al(III). Although the absolute concentrations of siderophore (relative to Desferal) varied slightly with the three metal assays, the pattern of responses to the presence of metals in the culture media was the same. Siderophore production was recorded with the CAS (Fe) assay for all isolates at their maximum culture density (Table 3). The presence of any of the metals in the culture media affected the production of siderophore. With all isolates, except H, the presence of added iron greatly decreased the siderophore concentration (Table 3). With isolate O no siderophore was detected in these high-iron cultures. Isolate H produced very low concentrations of siderophore under all culture conditions. The presence of gallium decreased the concentration of siderophore produced by isolates 37 and E1, whereas aluminium decreased the concentration of siderophore produced by isolate W2 (Table 3).

The assays used to distinguish the chemical structure of the siderophores were both apparently less sensitive than the CAS assay. There was also a problem with high background readings when assaying supernatants from the semi-defined medium. Results indicated that isolates H, E1 and W2 produced hydroxamate-type siderophores. However, supernatants from E1 cultures gave a slight positive response to the catecholate assay as well. For isolate O there was no clear response, culture supernatants giving slightly higher readings than controls with both assays. Isolates 15 and 37 both apparently produced catecholate-type siderophores, although the response from isolate 37 was very low. Results for hydroxymate tests on these two isolates are not available.

During growth of cultures in the defined medium, the pH generally decreased from 8.7 to 8.1. With the semi-defined medium the initial pH was considerably higher (10.6 to 11.0) and during the growth of some cultures the pH decreased as low as 7.5. However, for all isolates, siderophore production occurred when the pH was in excess of 7.5.

Discussion

One of the major problems encountered during this attempt to isolate alkalophilic siderophore producers was the maintenance of pH in batch culture. With media of high initial pH (pH 10.5–11.0) the commonly observed problem of pH drop due to culture growth is exacerbated by the absorption of CO_2 from the atmosphere (Grant and Tinall 1980). The use of automatic pH controllers would solve the problem but necessarily limits the number of simultaneous enrichments that can be made. The object of the work presented here was to demonstrate that a range of bacteria can be isolated from alkaline environments. It would be interesting in future work to investigate the use of chemostat cultures under pH control for such enrichment and isolation.

The universal siderophore assay of Schwyn and Neilands (1987) proved very useful and adaptable since it depends on the iron-binding ability irrespective of chemical structure. Thus, the same assay could be used for all isolates and comparisons made of their binding efficiencies relative to Desferal. Furthermore the assay was amenable to modification so that the binding of gallium and aluminium as well as ferric iron could be monitored. In comparison to the assays based on chemical structure, the CAS assay is considerably more sensitive. The use of these assays demonstrated that both types of siderophore structure were represented in the selection of isolates.

The ability of biologically synthesised siderophores to chelate gallium has been shown previously (Emery 1971; Menon et al. 1987). However, this study reveals for the first time that many isolates from high pH environments produce siderophores that are able to bind gallium and aluminium as well as iron. The binding of gallium is perhaps somewhat surprising since it has no known biological function, gallium (III) being incapable of reduction to gallium(II) in vivo (Emery and Hoffer 1980). The most likely explanation for the widespread existence of gallium-binding siderophores is the close similarity in size of the ionic radii of iron(III) and gallium (III) (Shannon 1976). Gallium present at a concentration representing at least a tenfold excess to iron had no apparent inhibitory effect on our isolates although when present at a similar concentration in low-iron medium it has been reported to diminish the growth yield of *Escherichia coli* (Hubbard et al. 1986).

The results presented in Table 2 reveal many different patterns of growth and siderophore production re-

lating to iron or gallium. In many cases the siderophore concentrations determined for gallium and iron were not equivalent. This result may reflect different affinities of one siderophore for the two metals or indeed the presence of more than one siderophore. Similarly the apparent lack of gallium toxicity may be due to lack of metabolic interference by gallium (III), or to the fact that siderophores able to bind gallium are produced in excess. Further investigations are required to resolve these questions. In the growth studies reported here the presence of iron greatly reduced siderophore production with all isolates except one (H), an observation in agreement with previous studies (Emery 1971; Fekete et al. 1983). It is interesting to note that the presence of gallium or aluminium also interfered with siderophore production by some isolates.

This study has shown that there are many potentially useful isolates capable of binding gallium whilst growing at an alkaline pH. However, many questions remain. It will be interesting to determine whether the siderophores produced by these organisms have novel structures related to operating in alkaline conditions. Furthermore, are these bacteria capable of transporting the gallium into the cell once it has been bound by the siderophores?

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