Effect of nutrient limitation on biofilm formation and phosphatase activity of a *Citrobacter* sp.

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A phosphatase-overproducing *Citrobacter* sp. (NCIMB 40259) was grown in an air-lift reactor in steady-state continuous culture under limitation of carbon, phosphorus or nitrogen. Substantial biofilm formation, and the highest phosphatase activity, were observed under lactose limitation. However, the total amount of biofilm wet biomass and the phosphatase specific activity were reduced in phosphorus- or nitrogen-limited cultures or when glucose was substituted for lactose as the limiting carbon source. Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM) showed differences in cell and biofilm morphology in relation to medium composition. Electron microscopy suggested that the differences in biofilm formation may relate to differential expression of fimbriae on the cell surface.

Keywords: attached cells, starvation, phosphohydrolase, confocal laser scanning microscopy, fimbriae

INTRODUCTION

Microbial biofilms have been used for a number of applications including the treatment of drinking water and wastewater (Bryers, 1994). The use of a naturally immobilized biofilm circumvents the need to artificially immobilize a pre-grown planktonic population. Biofilm produces a high concentration of biomass, allowing relatively small reactors, and short retention times (Janning *et al.*, 1995). The heterogeneous structure of natural biofilms can confer additional advantages, their surface being more irregular, and hence of a higher surface area, than that of an artificially immobilized cell population. Structural features, such as pores or channels (Costerton *et al.*, 1994; Wimpenny & Colasanti, 1997; Lewandowski, 1998) allow bulk fluid permeation throughout (de Beer *et al.*, 1994).

Understanding of the processes of biofilm formation remains incomplete. Factors such as the hydrodynamics of the bulk fluid (Kugaprasatham *et al.*, 1992; Peyton, 1996), the nature of the substratum (Dalton *et al.*, 1994; van Loosdrecht *et al.*, 1995), species composition

(Lawrence *et al.*, 1991) and nutrient availability (Huang *et al.*, 1994; Ohashi *et al.*, 1995; Peyton, 1996) influence biofilm formation, but it is still not clear exactly how these factors interplay, or which factors dominate. Therefore, the primary aim of this study was to determine the extent to which a single change in growth conditions affects the formation of a biotechnologically useful monospecies biofilm, excluding possible variables such as interspecies interactions and communication which are often observed (James *et al.*, 1995; Davies *et al.*, 1998).

The test organism, a *Citrobacter* sp. (NCIMB 40259) has been used for the bioremediation of heavy metals via the activity of an acid-type phosphatase enzyme (Jeong & Macaskie, 1999) which liberates HPO_4^{2-} from a suitable organic phosphate donor with the stoichiometric precipitation of metal cations (M^{2+}) as insoluble MHPO_4 at the cell surface (Macaskie *et al.*, 1995; Finlay *et al.*, 1999). Conditions promoting maximum biofilm growth may not necessarily produce maximum enzymic activity. Therefore phosphatase specific activity was investigated in parallel with morphological and quantitative studies of the biofilm with respect to the effect of nutrient limitation. The latter is known to promote changes in cell physiology and composition (Harder & Dijkhuizen, 1983; Breedveld *et al.*, 1995), but the

Abbreviations: CLSM, confocal laser scanning microscopy; EPS, extracellular polymeric substance(s); SEM, scanning electron microscopy; TEM, transmission electron microscopy.

consequences of these changes on biofilm formation and structure are largely unknown. Carbon limitation can promote cell adhesion to surfaces (Ellwood *et al.*, 1982), while the C:N ratio of the medium can affect species composition (Ohashi *et al.*, 1995), plasmid stability and the ratio of polysaccharide to protein content within biofilms (Haung *et al.*, 1994; Ohashi *et al.*, 1995). Upregulated alkaline phosphatase was visualized in phosphorus-limited biofilms (Huang *et al.*, 1998) but in contrast acid phosphatase production is regulated by the carbon status of the medium and the enzyme has a less clearly defined role (Jeong & Macaskie, 1999; Macaskie *et al.*, 2000). In this study *Citrobacter* sp. strain NCIMB 40259 was grown in a chemostat under carbon, phosphorus or nitrogen limitation and the phosphatase activity, cellular morphology and biofilm formation and structure were evaluated. The results are discussed with respect to factors affecting biofilm formation.

METHODS

Micro-organism and growth conditions. *Citrobacter* sp. NCIMB 40259 was used under licence from Isis Innovation, Oxford, UK. Biofilms were grown under lactose limitation in an air-lift reactor as described by Finlay *et al.* (1999) or, for comparison of growth under carbon, phosphorus or nitrogen limitation, in three parallel scaled-down (tenfold) reactors (working volume of 250 ml each). Dissolved oxygen (dO_2) and temperature (not less than 50% dO_2 and 30 °C, respectively) were monitored as previously (Finlay *et al.*, 1999). For subsequent analyses using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) glass cover slips (22 × 64 mm; Chance Propper) were suspended via silicone sealant in the 'down-flow' side of the reactor on a purpose-built silicone tubing support (Finlay *et al.*, 1999). The apparatus (acid washed and extensively rinsed prior to assembly) was sterilized by autoclaving (121 °C, 30 min). Inocula were taken from a stock plate (nutrient agar; Oxoid) and grown aerobically (30 °C; 24 h) in 25 ml of the appropriate medium, subcultured, allowed to grow for a further 12 h, added to the reactor aseptically and grown batchwise (24 h) before being switched to continuous culture (dilution rate, D , 0.12 h^{-1}) for a total of 160–170 h, unless stated otherwise. The basal salts medium (pH 7.4, with polypropylene glycol antifoam at 2.5 $\mu\text{l l}^{-1}$) was modified for carbon-, nitrogen- or phosphorus-limited chemostats as shown in Table 1. The ratio of different elements was

calculated on the basis of media described by Hambling *et al.* (1987) except that the phosphorus-limited medium was modified in order to allow the three media to support a comparable biomass density. All chemicals were from BDH/Merck. Outflow samples were collected for monitoring the OD_{600} , pH and phosphatase activity. Samples were plated on 2% (w/v) nutrient agar (Oxoid) and minimal-medium plates to check for culture purity and replica plated for confirmation on Simmons' citrate medium, comprising (g l^{-1}): NaCl, 5.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{NH}_4\text{H}_2\text{PO}_4$, 1.0; trisodium citrate, 2.0 (pH 6.8). The media were solidified with 2% (w/v) agar (Bacto agar; Difco) with 0.008% (w/v) bromothymol blue. Three independent experiments for each condition were performed unless otherwise stated, and quantitative data were acquired using at least 10 replicate samples from each fermenter. Details are given where appropriate.

Estimation of biofilm biomass. After each experiment the suspended cell culture and glass cover slips were removed from the reactor. Residual biofilm on the reactor walls was flushed free repeatedly with isotonic saline (8.5 g NaCl l^{-1}). Lack of residual biomass in the reactor was confirmed by staining biofilm-loaded and washed glass with acridine orange solution (0.005%, w/v) and viewing under UV illumination. The biofilm biomass was harvested by centrifugation in pre-weighed centrifuge tubes, washed once with isotonic saline and drained by inversion to obtain an estimate of wet weight harvested from a known bioreactor surface area of 0.0256 m^2 . The dry weight was obtained by drying a known amount of wet biofilm biomass to constant weight (95 °C for 3 d).

Assay of phosphatase activity. Phosphatase specific activity was measured by the release of *p*-nitrophenol (PNP) from *p*-nitrophenyl phosphate as described previously (Macaskie *et al.*, 1995, 2000; Jeong *et al.*, 1997; Finlay *et al.*, 1999; Bonthron *et al.*, 2000). One unit is defined as 1 nmol PNP released min^{-1} (mg bacterial protein) $^{-1}$ with the protein concentration measured using the CuSO_4 /bicinchoninic acid method (Sigma protein test kit TPR0562) and bovine serum albumin as the standard. For estimation of biofilm enzyme activity, samples were prepared by washing and resuspending the biofilm in isotonic saline and vortex-mixing (4 min) to break apart cell aggregates prior to assay.

Electron microscopy. Each sample was fixed immediately in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2, 4 °C, overnight). Dehydration was in a graded ethanol series, followed by critical-point drying with carbon dioxide. Samples were sputter-coated with gold prior to examination using a scanning electron microscope (Hitachi

Table 1. Composition of media (g l^{-1}) for three different nutrient-limiting regimes

Component	Lactose-limited	P-limited	N-limited
Tris (buffer)	12	12	12
KCl	0.62	0.62	0.62
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.063	0.063	0.063
$(\text{NH}_4)_2\text{HPO}_4$	0.96	0.01	0.104
$(\text{NH}_4)_2\text{SO}_4$	—	1.157	—
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.00032	0.00032	0.00032
KH_2PO_4	—	—	0.85
Lactose*	0.6	3.0	3.0

* For glucose-limited cultures the composition was identical to the lactose-limited medium except that the lactose was replaced with 0.6 g glucose l^{-1} . Media were adjusted to pH 7.24 with concentrated HCl.

2300; accelerating voltage 15 kV). Samples for negative staining for transmission electron microscopy (TEM) were washed three times in isotonic saline. The cell density of the sample was adjusted to $OD_{600} \sim 0.2$. The samples were attached to a carbon-coated copper grid by air fixation, stained with 10 μ l sodium phosphotungstate solution (5%, w/v, pH 7.3; 5 min), washed by brief dipping in distilled water (subsequently drawn off onto filter paper) and dried for 1 min under a lamp. Samples were viewed using a JEOL 1200 EX electron microscope (accelerating voltage 80 kV).

Confocal laser scanning microscopy (CLSM). Biofilm was removed from one side of cover slips by washing with 70% (v/v) ethanol. The silicone tubing was removed and the biofilm on the other side was stained with 0.005% (w/v) acridine orange for 5 min in the dark and washed three times in sterile isotonic saline. The biofilm was viewed under a Bio-Rad MRC 600 CLSM attached to a Nikon Diaphot microscope and an appropriate set of filters (488 nm excitation filter, 510 nm longpass filter and < 515 nm barrier filter). The alignments of the laser and monitor set-up were checked according to the manufacturer's instructions. The sample was observed under a $\times 40$ (dry: NA 0.75) and $\times 60$ (oil immersion: NA 1.4) lens, with the latter used for image acquisition. Use of an inverted microscope (sample viewed from the clean side of the cover slip, under oil), circumvented the need for a cover slip over the film, which would have compressed the biofilm structure. Five orthogonal (vertical) section images were taken at random from each sample using the 'Vertical Section' function of the instrument and saved onto a Zip disk (Iomega). Height measurements were made from these 'transsects', with each measurement taken vertically from the cover slip to the top of the biofilm. Twenty measurements were taken at regular distances (8.3 μ m apart) across the horizontal 'transsects'. Measurements, calibrated against fluorescent beads with a diameter of 15 μ m (Molecular Probes), were made using COMOS software as supplied. The area fraction (% coverage) was measured from a series of optical sections imaged at different depths at the same xy coordinates (horizontal planes at different depths: z series). Measurements were made using the BAND function of the COMOS software. The threshold value was set manually and retained for all sections in the same plane.

RESULTS

Growth of organism and phosphatase activity

Growth of the cultures under nutrient limitation is shown in Fig. 1. The final OD_{600} was similar for all three conditions [C limiting, 0.280 ± 0.040 ; P limiting, 0.290 ± 0.040 ; N limiting, 0.256 ± 0.010]; and the biomass measured as mg protein ml^{-1} was not significantly different (Table 2). Each culture was validated as being restricted for the required nutrient by sampling at steady state and confirming additional growth in batch culture when supplemented with appropriate individual medium components.

The highest phosphatase specific activity (in planktonic cells: culture outflow) was obtained under lactose limitation, with significantly lower activities under P- and N-limitation ($F_{2,6} = 25.6$; $P < 0.01$; Table 2). When limiting glucose was substituted for limiting lactose in additional chemostats the phosphatase specific activity was lower: 417 $nmol\ min^{-1}\ (mg\ protein)^{-1}$

Fig. 1. OD_{600} and pH of *Citrobacter* NCIMB 40259 grown in continuous culture under: (a) lactose limitation; (b) P limitation and (c) N limitation. The first arrow depicts change from batch culture to continuous culture. The second arrow shows the time at which biofilms or free cells were harvested for analyses. Open symbols, biomass content of liquid phase (OD_{600}); filled symbols, pH of culture outflow. The three shapes of symbols relate to three independent experiments. The biomass content and pH data were fitted to polynomial curves of the 4th and 2nd order, respectively, using Sigma Plot 5.0.

(Table 2), and was comparable to that of P-limited cells. This effect was confined to glucose; previous continuous cultures under glycerol limitation (Macaskie *et al.*, 1995; Jeong & Macaskie, 1999) gave a specific activity comparable to that of the lactose-limited cultures (Table 2). The phosphatase specific activity of harvested biofilm cells grown under lactose limitation was $\sim 850\ nmol\ min^{-1}\ (mg\ protein)^{-1}$ (Table 2), a reduction of approximately 50% compared to the cells in suspended culture. This contrasts with cells grown under glycerol limitation, where recovered biofilm-cells had a similar phosphatase activity to cells harvested from the culture outflow (P. Clark & L. E. Macaskie, unpublished). It is assumed that the ratio of true planktonic cells to sloughed or biofilm-detached cells in the respective media was the same in each case; differentiation between the two types of cell is not trivial and was not attempted,

Table 2. Effect of nutrient limitation on phosphatase activity and biofilm formation

All data were collected between 160 and 170 h for each experiment (arrowed in Fig. 1). Phosphatase activity was calculated on the basis of the protein content for each type of culture. Data are means $\pm$ standard errors from three independent chemostats except in the case of glucose limitation, where the mean of two chemostats is shown. ND, Not determined.

Limiting nutrient	Biomass (mg protein ml ⁻¹) of planktonic culture (at OD ₆₀₀ 1.0)	Phosphatase specific activity (units)		Wet weight of biofilm harvested (g)
		Cells from culture outflow	Cells from biofilm	
Lactose	438 $\pm$ 37	1971 $\pm$ 357	851 $\pm$ 99	3.19 $\pm$ 0.21
Glucose	ND	417	ND	0.31
Phosphorus	459 $\pm$ 21	401 $\pm$ 77	ND	0.35 $\pm$ 0.15
Nitrogen	421 $\pm$ 19	124 $\pm$ 47	ND	0.17 $\pm$ 0.18

but the similar activity between biofilm and free cells in the glycerol-limited culture (above) suggests this to be the case. The negligible biofilm yield under P-, N- and glucose limitation precluded a comparative assay of phosphatase activity of biofilm-cells. Previous studies have localized phosphatase activity within biofilms using fluorochromes (Huang *et al.*, 1998) but preliminary tests in this study showed no clear localization; furthermore, diffusion of the substrate into deeper layers of the biofilm could not be proved, and this approach was not pursued further.

Biofilm formation in nutrient-limited continuous culture

Biofilm was apparent on the vessel walls after 96 h under lactose limitation, but not under N or P limitation. Table 2 confirms that the wet weight of biofilm harvested from the known surface area of the reactor was significantly higher ($F_{2,6} = 101$; $P < 0.01$) for lactose-limited cultures. The dry weight of biofilm was estimated in parallel for each experiment; the wet weight/dry weight ratio was 10:1, in accordance with previous studies using batch-cells (P. Yong & L. E. Macaskie unpublished). The carbon source was also important; glucose limitation produced a mean biofilm biomass of 0.31 g, which was only 10% of that under lactose limitation (3.2 g) and was comparable to P-limited cultures (Table 2). In an extended experiment a culture grown under N limitation (with lactose) for 15 d gave 0.47 g of biofilm biomass, i.e. the amount of biofilm had more than doubled, but was still 86% less than for lactose-limited cells.

Examination of surface features of biofilm and planktonic cells

Cells were negatively stained and examined by TEM. A minimum of 50 cells was observed from each sample. Almost all biofilm cells (91%) from the lactose-limited medium had numerous appendages (fimbriae: Sharon, 1984) covering the whole cell surface (Fig. 2a) but only

55% of planktonic cells from the same chemostat displayed these. Only a minority of either type of cells grown under P, N or glucose limitation (< 30%) showed appendages. Each cell displayed a large number of fimbriae or none (Fig. 2a, b); appendages were divisible into clearly visible large structures and an indistinct fuzzy layer covering the cell (Fig. 2c), probably comprising 'curli' (Olsen *et al.*, 1989; Fig. 2c, inset).

Examination of biofilms by electron microscopy

When viewed using SEM lactose-limited cultures showed a thick, nearly confluent biofilm comprising a base layer of cells with outgrowing 'stacks' (Fig. 3a, arrowed) and visible (collapsed) extracellular material, cross-linking the cells and forming an integral part of the structure (Fig. 3b, inset). Corresponding cover slips withdrawn from P- and N-limited cultures showed a thin patchy monolayer (Fig. 3d, g, respectively) with, under P-limitation, small microcolonies and occasional long filaments (20–30 μ m). Replica-plating of nutrient-agar-grown colonies from the culture onto selective citrate medium showed growth in all cases (500 colonies examined), confirming culture purity. More generally, P-limitation gave short rods 1–3 μ m in length (Fig. 3f), while C-limitation promoted formation of uniform short filaments (Fig. 3c). N-limitation gave short rods of less than 1 μ m (Fig. 3i) and no variation in cell length (Fig. 3h); these cells, unlike their P-limited counterparts (Fig. 3e), did not form substantial microcolonies.

Biofilm measurement and structural analysis using CLSM

Preliminary studies showed that biofilm viewed under CLSM closely matched that seen under SEM (Finlay *et al.*, 1999). Examination of lactose-grown biofilms using CLSM showed that cells in immediate contact with the cover slip were oriented horizontally whilst those within the stacks were aligned vertically (not shown). Random orthogonal sections from a common baseline through biofilms grown under the three different conditions

Fig. 2. TEM images of negatively stained whole sample mounts of *Citrobacter* sp. (a) Representative cell from lactose-limiting chemostat; (b) corresponding cell from a P-limiting chemostat and (c) magnified portion of cell shown in (a) showing fimbriae and detail of cellular attachment. Bars, 200 nm. The inset in (c) shows a magnified area of the cell surface to show detail of 'curli'.

clearly confirmed differences in biofilm thickness (Fig. 4). At least 300 measurements (100 measurements taken from three replicate chemostats) were obtained from biofilms grown under each set of C- (lactose-), P- and N-

limiting conditions (Table 3). The standard deviation and range of these measurements provide a measure of the variation or heterogeneity in the biofilms; the smaller the SD, the more homogeneous the biofilm. The large SD values show that the biofilms were heterogeneous. The mode and median values, which are not as sensitive to a few large measurements as mean values, suggest a structure comprising a base layer of between about 8 and 20 μm with large outgrowing stacks, in the lactose-limited cultures only, of up to 100 μm in length (120 h biofilms; see Fig. 4). The minimum height measurements show that, despite the heavy biofilm produced by lactose-limited cells, areas of the substratum were still exposed to the bulk fluid in accordance with currently accepted models for biofilm structure (Costerton *et al.*, 1994; Wimpenny & Colasanti, 1997). Biofilms (144 h) from glucose-limited cultures comprised a monolayer of rod-shaped cells with isolated colonies, some of which protruded from the surface forming 'stacks' (Fig. 5b). The depths of lactose-limited biofilms grown on cover slips were measured as a function of time (Table 4); the thickness of the 144 h biofilm precluded detailed structural analysis using CLSM and detailed analyses were done using 120 h biofilms (Table 3). The 'stack' format was confirmed using SEM (Fig. 5c) and complementary CLSM (Fig. 5d), and further analyses aimed to obtain quantitative information (Table 3).

A series of *xy* optical sections was obtained, expressing the biofilm coverage of the field area in terms of the % void. This is shown in Fig. 6 as the decrease in percentage biofilm coverage with increasing distance from the substratum. At the substratum the biofilm coverage was 70%, confirming that pores and channels extended vertically through the film. These are not visible from above, being occluded by the stacks, but can be seen clearly from sagittal images (Fig. 5c, d). The percentage coverage decreased exponentially with the distance from the substratum (Fig. 6), i.e. $y = -8.0851 + 78.0643 e^{-0.0232x}$ ($r^2 = 0.999$), suggesting a regular structure. The void area of the top of the biofilm, i.e. above 50 μm , increased with a smaller loss of coverage per unit of depth (Fig. 6) compared to that nearer the substratum, i.e. below 50 μm , probably attributable to the differences in 'stack' and base layer structure. The 'stacks' were dispersed randomly throughout the structure; the dispersion index (sample variance/sample mean) was 0.93. Accordingly the dispersion of clusters showed good agreement with the Poisson model of dispersion ($\chi^2 = 3.64$).

DISCUSSION

Citrobacter NCIMB 40259 grown under three different nutrient limitations had comparable planktonic population densities at steady-state. It is possible that a portion of the planktonic cultures contained cells that had emerged from the biofilm. This differentiation is difficult. However, biofilm thickness measurements of the lactose-limited cultures suggest that the depth was still increasing exponentially at 144 h with no apparent

Fig. 3. SEM of 144 h (see Fig. 1) *Citrobacter* biofilms grown under conditions of lactose limitation (a, b, c), P limitation (d, e, f) and N limitation (g, h, i). Bars, 200 μm (a, d, g), 20 μm (b, e, h), 5 μm (c, f, i). In the case of (c) the field of view was selected at the edge of the slide in order to see single cells.

Fig. 4. CLSM digital images showing orthogonal view (xz axis) of *Citrobacter* biofilms formed under three different nutrient regimes corresponding to those shown in Fig. 3 (a, b, c, lactose, P- and N-limiting, respectively). All images were obtained at identical magnification and are shown from the substratum (black area). Bars, 25 μm . Images were obtained with a Bio-Rad MRC 600 CLSM using a $\times 60$ oil immersion lens, saved as PIC files and converted to TIFF files using the software Confocalist. Lactose-limited biofilms were harvested after 120 h of growth because 144 h biofilms were too thick to give clear images; P- and N-limited biofilms were harvested after 144 h.

slowing attributable to biofilm attrition, and the design of the vessel (air-lift circulation with protection of the biofilm from the bubble mass: Finlay *et al.*, 1999) produced minimal shear. A pure biofilm population could have been obtained by increasing the dilution rate to obtain washout of the planktonic cells but when $D > \text{maximum specific growth rate } (\mu_{\text{max}})$ (i.e. washout) the biofilm would probably cease to be limiting for the appropriate nutrient. Negligible biofilm formation oc-

curred under non-limiting nutrient conditions (batch cultures), making comparison between non-limited and limited cultures difficult. As a compromise the status of the medium was changed from C-limiting to C-sufficient by altering the nature of the limiting nutrient from C to P or N at a constant value for μ (where $D = \mu$).

Substantial biofilm growth was observed under lactose (this study) or glycerol (Macaskie *et al.*, 1995) limitation but not in P-, N- or glucose-limited cultures. Biofilm formation is associated with the synthesis of extracellular polymers, which is energetically demanding and carbon-expensive (Chakrabarty, 1996), and cells usually attempt to conserve available carbon for essential functions (Harder & Dijkhuizen, 1983), which mainly involves minimizing the diversion of substrate carbon into extracellular polymeric substances (EPS) (Tempest & Wouters, 1981). It was shown previously that the composition of the EPS of this organism and also the degree of metal-ion-mediated cross-linking are dependent on the growth medium (Bonthrone *et al.*, 2000). In this study, although negligible biofilm was formed under P and N limitation the initial event in biofilm formation, *viz.* cell-substratum attachment (Korber *et al.*, 1995), did occur. Biofilm continued to accumulate under N-limitation over a longer duration, but the amount was still negligible compared with that obtained under lactose limitation. With limiting glucose, the amount of biofilm was decreased, suggesting that the nature of the limiting carbon source is also important. A glucose-repressive effect may occur, even under prolonged starvation conditions, an observation that would warrant further study.

An electron microscope study of biofilm and planktonic cells grown under the three conditions showed morphological differences. Bacterial cell morphology varies in response to environmental signals, with a reduced cell size commonly promoted as a response to starvation (Mueller, 1996). Filamentous *Pseudomonas* were reported in biofilms (Jensen & Woolfolk, 1985), possibly promoted by O_2 -limitation and turbulent conditions. The filamentous cells seen under P-limitation (this study) resemble the elongated *Escherichia coli* and *Salmonella typhimurium* swarmer cells described by Harshey & Matsuyama (1994). An increasing number of identified species produce swarmer cells, a phenomenon which also appears to be related to surface colonization (Harshey, 1994). However, in our study colonization under P-limitation was poor, with only a small number of long cells; furthermore there was no evidence (Fig. 3) to suggest that microcolony formation was related to long cell occurrence. Electron microscopy showed that the cell surfaces appeared to alter under the different nutrient restrictions, with populations differing in their expression of cell surface appendages, resembling fimbriae (Sharon, 1984), some of which were designated as a special class ('curli'), which are implicated in surface colonization and biofilm formation (Olsen *et al.*, 1989). There is interest in the regulation of fimbrial expression as these structures are also associated with virulence (Curtiss & Kelly, 1987; Vidal *et al.*, 1998), their presence

Table 3. Summary of thickness measurements of *Citrobacter* biofilms using CLSM

Parameter*	Limiting nutrient		
	Lactose (120 h biofilm)	P (144 h biofilm)	N (144 h biofilm)
Mean (μm)	26.0	2.1	0.8
SD of mean (μm)	21.7	3.7	1.4
CV† (%)	83	176	175
Mode† (μm)	8.6	0	0
Median† (μm)	19.6	1.3	0.5
Maximum (μm)	122.0	35.4	0
Minimum (μm)	0	0	0
n†	320	300	300

* Measurements were made on *Citrobacter* biofilms grown on glass cover slips (average of three chemostats).

† CV (coefficient of variation) is the ratio of the standard deviation (SD) to the mean. Mode is the most frequently occurring, or repetitive, value in a range of data. Median is the middle observation in a set of observations which have been ranked in magnitude and *n* is the number of measurements.

Fig. 5. (a, b) CLSM digital images of 144 h *Citrobacter* biofilms grown under lactose (a) and glucose (b) limitation. Both biofilms were stained with acridine orange and images (z series) were obtained at identical magnification with a Bio-Rad MRC 600 CLSM. Separate images were collected at 10 μm depth intervals through the biofilm and were projected as one image. Images were saved as PIC files and converted to TIFF files using Confocalist. Bars, 25 μm . (c, d) Further analysis used 120 h *Citrobacter* biofilm grown under lactose limitation and examined using (c) SEM (30° tilt; bar, 50 μm); (d) corresponding CLSM image showing orthogonal view (xz axis) of the biofilm stained with acridine orange (bar, 25 μm). Clusters of biomass, typically mushroom shaped, and base layers are visible.

facilitating attachment to the host tissues. Regulation of fimbrial expression is strongly affected by environmental conditions, e.g. temperature, external pH, osmolarity

and nutrient status (van der Woude *et al.*, 1989; Schmoll *et al.*, 1990; Edwards & Schifferli, 1997; Vanmaele & Armstrong, 1997; Xie *et al.*, 1997). In the case of ‘curli’

Table 4. Depth measurements of *Citrobacter* biofilm grown on glass cover slips, under lactose-limited conditions, as a function of time

Data are means $\pm$ SD from three independent chemostats. Each datum represents the mean of 20 measurements taken 8.3 μ m apart across five horizontal transects (see text).

Time (h)	Mean depth (μ m)	Maximum depth (μ m)	Minimum depth (μ m)
72	3.0 $\pm$ 0.1	10.5 $\pm$ 0.5	0
96	19.6 $\pm$ 2.7	45.3 $\pm$ 1.4	0.8 $\pm$ 0.6
120	26.1 $\pm$ 2.1	121.8 $\pm$ 19.8	2.4 $\pm$ 1.2

Fig. 6. Biofilm coverage as a function of biofilm height from the substratum. CLSM was used to obtain a series of optical sections of 120 h *Citrobacter* biofilms. Each value represents the mean value of 10 measurements taken from fields of view at the same depth in the biofilm sample. The measurements were made using the BAND function of the COMOS software. Standard errors are not shown but were within 5%.

expression the subunit gene *csgA* is regulated by the RpoS sigma factor (Olsen *et al.*, 1993) and also via the OmpR component of the two-component EnvZ/OmpR sensor-regulator, concluded by analysis of adhesive mutants of *E. coli* selected under continuous culture (Vidal *et al.*, 1998). Thus, it seems possible that expression of fimbrial adhesins on the cell surface is responsible for the various degrees of biofilm observed here. Accordingly mutants of *E. coli* and *Pseudomonas aeruginosa* unable to form biofilm on polyvinyl chloride (PVC) lacked the ability to produce type I and type IV fimbriae, respectively (Pratt & Kolter, 1998; O'Toole & Kolter, 1998), while *Salmonella enteritidis* defective in biofilm formation on stainless steel and teflon failed to produce thin, aggregative fimbriae (designated SEF 17: Austin *et al.*, 1998). These studies suggest that more than one fimbrial type is involved in biofilm development (Stickler, 1999) and, indeed, both the large fimbriae and the curli were absent in the P- or N-limited *Citrobacter* cells (this study, Fig. 3b). The observation that fimbrial expression appears to be reduced with glucose as the carbon source, even when cells are C-restricted, suggests a level of control that would warrant future investigation.

Cell surface fimbrial expression does not preclude the possibility that the cell wall composition, or the EPS produced under lactose limitation, may differ in a way that promotes cell-to-cell or cell-to-substratum adhesion. Cell surface composition and chemistry are functions of the cellular environment (Herbert, 1961), with many differences reported for cells grown under P and C limitation (Zhan *et al.*, 1991; Breedveld *et al.*, 1995; Bonthron *et al.*, 2000).

Lactose-limited biofilm shares many features with other biofilms. Wimpenny & Colasanti (1997) reviewed three different conceptual models of biofilm structure: the dense biofilms model, the heterogeneous mosaic model (Keevil & Walker, 1992) and the water channel model (Costerton *et al.*, 1994) by which most biofilms may be described. Of these, the last is the most suitable to describe the *Citrobacter* lactose-limited biofilm, the thickness of which corresponds well with depth measurements reported for other biofilms, e.g. that of a *Klebsiella pneumoniae*/*P. aeruginosa* mixed population (Murga *et al.*, 1995), although the *Citrobacter* biofilm is thick compared to *Pseudomonas* biofilms ($\sim 30 \mu$ m: Stewart *et al.*, 1993; Murga *et al.*, 1995) and is more comparable to the biofilm of *Klebsiella pneumoniae* (100 μ m: Murga *et al.*, 1995).

In contrast to the lactose-limited biofilm the structure of the glucose-limited *Citrobacter* biofilm at the same age (Fig. 5) resembled the heterogeneous mosaic biofilm model (Keevil & Walker, 1992); the majority of the surface was colonized by single cells and discrete colonies, with some growing into stacks. The effect of prolonged culture and the extent to which glucose-mediated regulation plays a role were not investigated further.

In order to attempt to explain the nutrient-limitation-dependent variation in biofilm formation it was considered whether the increased biofilm production was related to increased EPS production. Tait *et al.* (1986) observed that high C:N and C:P ratios in the medium were associated with high levels of polymer production. However, no difference was found between the amount of polymer extracted from planktonic cells in the culture outflow in the three media (extraction and quantitation methods were as described by Bonthron *et al.*, 2000). Hence, there is probably no association between the

level of EPS production and the extent of biofilm formation by this strain (but note that there was insufficient biofilm for comparison of EPS of attached cells). In contrast, other studies (Bontrone *et al.*, 2000) showed that more EPS was produced from lactose-grown than from glycerol-grown cells in batch culture and that the content of cross-linking cations was higher. Also, the phosphate species of the lipopolysaccharide was different in the two types of cell, determined from the ^{31}P NMR spectra attributed to phosphate groups of the lipid A component of the lipopolysaccharide and differentiated from phospholipids by the NMR spectra (Bontrone *et al.*, 2000). The same study showed that material extracted from planktonic phosphate-limited cells from a chemostat gave a different ^{31}P NMR spectrum, with only one major phosphate species instead of three as seen under C-limitation. Clearly there can be some differences in the structure of the extracellular LPS and EPS but since N- and P-limited cells gave similar overall yields of EPS and similar biofilms (this study) differences in the EPS structure and composition are unlikely to play a major role.

A major objective of this work was to achieve good biofilm growth commensurate with high phosphatase activity, since this allows the biofilm to accumulate heavy metals (Macaskie *et al.*, 1995; Finlay *et al.*, 1999); some of the phosphatase enzyme is 'tethered' to cell surface materials (Macaskie *et al.*, 2000). The phosphatase (PhoN) activity of this strain was investigated previously (e.g. Hambling *et al.*, 1987; Jeong & Macaskie, 1999; Finlay *et al.*, 1999) and this study shows that activity was affected significantly by nutrient limitation, with the highest activity observed under lactose limitation; the values reported for lactose-limited planktonic cells in the outflow (Table 2) are in good agreement with previous studies (Finlay *et al.*, 1999). Variation in phosphatase activity between planktonic cells in replicate experiments was noted by Hambling *et al.* (1987) and these authors reported a similar difference in activity between C- and P-limited cells (75% decrease in the latter). The effect of N limitation has not been previously described. The activity of PhoN in *Salmonella* is under the control of the *phoP/phoQ* sensor-regulator system (Miller *et al.*, 1989) and in the present strain is regulated by both C and P limitation (Butler *et al.*, 1991; discussed in Jeong & Macaskie, 1999) with a transient repression in batch cultures seen under glucose addition (Butler *et al.*, 1991). The results presented here support this finding, as substitution of the lactose carbon source by glucose gave lower planktonic cell phosphatase activity (Table 2).

Microbial activity can be affected by attachment to a surface (Fletcher, 1991). Most examples describe enhanced activity but, conversely, in some cases surface attachment reduces cell activity (Bright & Fletcher, 1983; Gordon *et al.*, 1983). Here, *Citrobacter* phosphatase activity was lower in the lactose-limited biofilm cells as compared to planktonic cells although a previous study using glycerol-limited cells gave a similar activity in both populations (P. Clark & L. E. Macaskie,

unpublished). Estermann *et al.* (1954) reported that phosphatase activity was depressed by the addition of a surface and the present results suggest that this response is modified by the nature of the carbon source. It could be argued that this is an effect of local pH but no pH gradients were found in lactose-limited biofilms using pH microelectrode probes although lactose-pre-grown biofilms developed a pH gradient (pH 7.0 in the bulk fluid; pH 5 at the surface of the substratum) when subsequently grown-on in P- or N-limited media (Allan *et al.*, 1999). Low pH was associated with decreased phosphatase production in planktonic cells (Jeong & Macaskie, 1999). Although the regulation of *phoN* is under the control of the *phoP/phoQ* sensor-regulator system (Miller *et al.*, 1989) this study does not attempt to address the question of whether the stimulus is nutrient restriction or is an effect of pH.

This study raises a number of important questions regarding biofilm regulation. It shows that the balance and composition of the medium play a role in determining whether a cell population forms a biofilm or not, and extends previous studies suggesting that phosphatase activity is regulated according to the nutrient conditions and/or pH. Both enzyme activity and biofilm formation are influenced by environmental conditions (correlation of biofilm biomass with planktonic cell phosphatase specific activity, $r^2 = 0.988$), implying that the genes responsible for both functions (and also, possibly, the production of fimbriae) may be organized within the same stimulon (a stimulon refers to all the operons responding together to an environmental stimulus, regardless of how many regulons and modulons may be involved: Neidhardt *et al.*, 1990). Various studies suggest that fimbrial expression is associated with biofilm formation; fimbriae are known to be involved in attachment (Low *et al.*, 1996; Edwards & Schifferli 1997; Goluszko *et al.*, 1997) and biofilm development (Austin *et al.*, 1998; O'Toole & Kolter, 1998; Pratt & Kolter, 1998) and are likely to be involved in the mechanism of biofilm formation in the present case. Previous studies have shown that regulation of fimbrial production is affected by carbon source, nitrogen source (Edwards & Schifferli, 1997), pH (Low, 1996) and temperature (Xie *et al.*, 1997). Edwards & Schifferli (1997) have proposed a model which describes colonization of the mammalian gut by enteric bacteria in terms of fimbrial expression and the effects of host environment. This model may warrant further study in the context of biofilms of environmental or biotechnological importance, both as carriers of biotechnologically important enzymes and also as a way to present the maximum biocatalytic surface prior to developing mathematical models of the biofilm surface and integration into existing bioreactor process models.

NOTE ADDED IN PROOF

Strain NCIMB 40259 has recently been reassigned to the genus *Serratia* (Pattanapitpaisal *et al.*, 2001).

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