Iron chelator, exopolysaccharide and protease production in *Staphylococcus epidermidis*: a comparative study of the effects of specific growth rate in biofilm and planktonic culture

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The growth rate of *Staphylococcus epidermidis* was controlled for populations growing as a biofilm and perfused with supplemented, simple-salts medium. Production of iron chelators, extracellular protease and exopolysaccharide (EPS) by these populations was assessed as a function of specific growth rate and compared to that by planktonic populations grown in the same medium within a chemostat. Perfused biofilms increased their iron chelator and protease production with increasing growth rate. Chemostat populations decreased their production of iron chelators with increasing growth rate, whilst showing much enhanced production of proteases at intermediate growth rates (μ 0.15–0.25 h⁻¹). Production of iron chelator and protease was generally 2–50 times higher by biofilms than by planktonic populations. EPS production was low and relatively unaffected by growth rate for the chemostat cultures (about 0.2 μ g per unit cell mass) but high for the attached biofilms, particularly at slow growth rates (about 4 μ g per unit cell mass). EPS production within the biofilms decreased markedly with increasing growth rate. At growth rates of 0.35 h⁻¹ and above, the levels of EPS for biofilms and planktonic populations were equivalent. The results of this study clearly indicate that growth as a biofilm markedly influences extracellular virulence factor production by *S. epidermidis*.

Keywords: *Staphylococcus epidermidis*, biofilms, exopolysaccharide, growth rate, virulence factors

INTRODUCTION

Staphylococcus epidermidis is a ubiquitous skin commensal of man and animals and an opportunistic pathogen associated with indwelling medical devices such as cardiac valves, prosthetic joints and pacemaker leads (Gristina *et al.*, 1987; Marrie *et al.*, 1982; Fidalgo *et al.*, 1990). In such nosocomial infection, the organism is generally found as a biofilm attached to the surface of a device. Biofilms are protected from host defences by diffuse exopolymer slime layers (glycocalyx) of bacterial origin (Johnson *et al.*, 1987; Gray *et al.*, 1987). These slime layers also protect the sessile population from any major fluctuations in the macro-environment (Costerton *et al.*, 1987) and from the

therapeutic effects of antibiotics such as tobramycin (Evans *et al.*, 1990; Anwar *et al.*, 1989) and vancomycin (Evans & Holmes, 1987).

The biofilm mode of growth is recognized as being of prime importance in the establishment and maintenance of bacterial populations within a wide variety of natural habitats, including infections and colonization of medical devices (Gristina *et al.*, 1987; Marrie *et al.*, 1982). Resistance of device-associated infections has been variously attributed to failure of antibiotics to penetrate the glycocalyx (Nickel *et al.*, 1985), slow growth rates within nutrient-deprived biofilms (Brown *et al.*, 1988) and/or to innate properties of adherent cells (Evans & Holmes, 1987). In this respect, close proximity of surfaces, whether chemically and nutritionally inert, nutritious and/or associated with other living cells, causes the expression of physiologies distinct from those of suspension cultures in the laboratory (Marshall *et al.*, 1971; Gilbert *et al.*, 1990;

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Abbreviation: EPS, exopolysaccharide.

van Loosdrecht *et al.*, 1990). Extracellular polysaccharide production is often derepressed (Deretic *et al.*, 1989), extracellular enzymes may be influenced (Giwerzman *et al.*, 1991), and at a genomic level recent work has noted the presence of 'touch-promoters' (Dagostino *et al.*, 1991).

Often such studies fail to take into consideration the effect of growth rate on the physiology of the organism and may fail to distinguish between characteristics unique to a biofilm mode of growth and those unique to slow growth (Brown *et al.*, 1988). Growth of bacterial biofilms at controlled growth rates (Gilbert *et al.*, 1989; Duguid *et al.*, 1992a) and comparisons with chemostat culture have allowed these separate influences to be assessed for a number of Gram-negative (Evans *et al.*, 1990, 1991) and Gram-positive (Duguid *et al.*, 1992a, b) pathogens. Such studies have identified not only effects associated with adherence *per se* but also an overriding influence of specific growth rate and nutrient deprivation (Brown *et al.*, 1990; Gilbert *et al.*, 1990). Use of these techniques has not only shown reduced growth rates in biofilms to be a major contributor to their recalcitrance but also that cell division cycle modulates dispersal of the cells (Allison *et al.*, 1990a, b) and susceptibility to quinolone (Evans *et al.*, 1991) and aminoglycoside antibiotics (Evans *et al.*, 1990).

The present study develops such approaches to growth-rate control of Gram-positive biofilms and examines the separate influences of growth rate and adherence upon the production of extracellular virulence factors by *S. epidermidis*.

METHODS

Bacterial strain. *Staphylococcus epidermidis* NCTC 11047 was used throughout. Stock cultures were maintained on Nutrient Agar (Oxoid CM 3) slants at room temperature in a darkened cupboard. The identity of the strain was confirmed by using the criteria and methods described by Schliefer & Kloos (1975).

Continuous culture. Continuous cultures were established, at 37 °C, utilizing a glass fermenter (1 litre, jacketed continuous-culture vessel, 500 Series, LH Fermentation), a peptone-supplemented minimal-salts medium (Duguid *et al.*, 1992a) and the methods described by Gilbert & Brown (1978). The medium contained sufficient carbon source (glycerol, 10 mM) to allow batch growth to a stationary-phase OD₄₇₀ of 1.0 with all other nutrients [(NH₄)₂SO₄, 6 mM; MgSO₄, 0.5 mM; KCl, 13.5 mM; KH₂PO₄, 28 mM; Na₂HPO₄, 72 mM; 1 mg thiamin l⁻¹; 0.5 mg biotin l⁻¹; 0.5 g Peptone P l⁻¹ (Oxoid L49)] present to excess and the medium buffered to pH 7.4. The medium contained no added iron other than that present as trace contaminants. Supplementation of this medium with iron gave an increased growth rate in batch culture, but no increase in stationary-phase cell-density. Whilst the medium restricts the extent of cell growth through carbon-source availability it is therefore also restrictive in iron and might cause the cells to express high-affinity iron-uptake systems.

Biofilm culture. Growth-rate-controlled biofilm cultures of *S. epidermidis* were established on cellulose acetate membranes according to the methods described by Gilbert *et al.* (1989) and Duguid *et al.* (1992a). Mid-exponential-phase cultures of *S. epidermidis* (50 ml, 1 × 10⁸ cells ml⁻¹) were collected by pressure filtration (6 p.s.i., 41.4 kPa) onto the surfaces of 0.22 µm pore-

size cellulose acetate membranes (47 mm diam., Millipore). The membranes were removed from the pressure-filtration device (Millipore) and inverted into the base of the modified fermenter (Gilbert *et al.*, 1989). The membranes were immediately perfused with fresh medium from their sterile sides at various controlled rates. Biofilms, which developed on the underside of the membranes, were shown to be relatively constant with respect to cell number (1–2 × 10⁹ cells per membrane) over 100 h. Steady-state flow rates of between 10 and 200 ml h⁻¹ were found to govern the rate of evolution of dispersed cells in the perfusate and thereby the specific growth rate of the population. At flow rates of > 180 ml h⁻¹, growth rates were maximal and at a value equivalent to $\mu_{\max}$ for the medium ($\mu_{\max} = 0.42$ h⁻¹). Cultures were equilibrated for 40 h, at steady state, prior to collection of perfused medium/biofilms. Steady-state biofilms were removed from the fermenter, cut into quarters and placed in sterile phosphate-buffered saline (0.1 M, pH 7.0). Samples were vigorously shaken for 10 min using a Griffin flask shaker. Preliminary experiments had determined that this released more than 99% of the cells from the membrane and that the majority (> 99%) were in suspension as single cells. Growth rates were calculated in each instance, with a knowledge of medium flow rate, from viable counts performed on the perfusates and resuspended biofilm (Duguid *et al.*, 1992a).

Iron chelators. Levels of iron chelators were assayed in culture supernatants according to the methods of Schwyn & Neilands (1987). All glassware was washed thoroughly using a mild detergent (Lipsol, LIP), rinsed and soaked in consecutive baths of EDTA (0.01%, 24 h) and HCl (0.1 M, 24 h). Soaking was followed by four consecutive rinses in reverse-osmosis water. Viable counts were performed upon freshly collected spent medium eluted from the chemostat and biofilms at each steady-state growth rate. Spent culture medium was then collected onto ice for 1 h for both perfused biofilms and chemostat cultures. Samples were centrifuged (1000 g, 15 min) and supernatant fluids reduced in volume by 90% by freeze-drying. Equal volumes of the concentrate and reagent were mixed and left at 20 °C for 1 h. The reagent consisted of Cetrimide USP solution (10 mM, 6 ml), distilled water (30 ml), iron chloride (1 mM in 10 mM HCl, 1.5 ml) and chrome azurol S (2 mM, 7.5 ml). These were mixed together and anhydrous piperazine (4.31 g dissolved in 20 ml acidified water) added. The volume was adjusted to 100 ml and sulfosalicylic acid (101.6 mg) dissolved within it.

The A_{630} was related to that of similarly treated samples of fresh culture medium. The iron chelator content of the samples was given by the reduction in A_{630} of the samples and was related to viable cell count.

Protease assay. Protease activity within concentrated culture supernatants was assayed using heat-denatured casein as the substrate and a method based on that described by Wretling & Wadstrom (1977). Aliquots of concentrated culture supernatant (1.0 ml) were warmed to 37 °C and added to the reaction mixture [2 ml 1.5 g heat-denatured casein l⁻¹ in phosphate-buffered (0.05 M, pH 7.4) calcium chloride] and incubated at 37 °C for 1 h. Perchloric acid (3 ml, 1 M) was added to stop the hydrolysis and the A_{280} read on a spectrophotometer. Protease activity was calculated as the A_{280} increase and related to viable count.

Exopolysaccharide (EPS) assay. EPS production was quantified using the methods described by Dall & Herndon (1989). Total counts were made, using a haemocytometer slide, on test suspensions taken directly from the chemostat and prepared from steady-state biofilms at various growth rates. Biofilms were dispersed from their cellulose-membrane supports by vigorous shaking (10 min), as described above.

Volumes of resuspended biofilm and/or planktonic culture (5 ml, 1×10^8 cells ml⁻¹) were centrifuged (1000 g, 15 min) in a Beckman J2-21 centrifuge. The resultant pellets were resuspended in sterile saline (1 ml) and sonicated (2 min, 60 W power) using a Soniprep 150 (MSE). The samples were further centrifuged (1000 g, 10 min) to remove cells, and the supernatant added dropwise to absolute alcohol (10 ml, 4 °C). After 24 h at 4 °C, the precipitated EPS was collected by centrifugation (10000 g, 20 min), resuspended in sterile water (1 ml), mixed with conc. H₂SO₄ (77%, 7 ml) and transferred to a boiling tube in an ice bath for 10 min. Cold tryptophan (1%, w/v; 1 ml) was added and the tubes heated in a boiling bath for 20 min to effect hydrolysis. Acid hydrolysis of EPS produces a furan which condenses with the tryptophan to produce a coloured product. This may be quantified, after cooling, by measuring A_{500} . Calibration curves were prepared against standard dextran solutions. Preliminary experiments had determined that solutions of hexose and pentose sugars, disaccharides, DNA and protein did not interfere with the assay. All determinations were performed in triplicate upon a minimum of two separate samples. Results were expressed as dextran equivalent units per 10^8 cells.

RESULTS AND DISCUSSION

Effect of specific growth rate on production of siderophores and protease enzymes

The effects of specific growth rate upon the production of protease enzymes and siderophores by planktonic and biofilm cultures of *S. epidermidis* are presented in Figs 1 and 2, respectively. Unlike batch cultures, in continuous culture secondary metabolites and extracellular products are subject to constant dilution by the addition of fresh medium. At steady states, production of such factors by

Fig. 1. Effect of specific growth rate upon the production of protease by adherent biofilms ($\blacktriangle$) and planktonic populations ($\blacksquare$) of *S. epidermidis* grown in a peptone-supplemented simple-salts medium containing no added iron.

the resident population equals their rate of removal/dilution. In chemostats the dilution effect is readily calculated from a knowledge of the culture volume and the flow rate of fresh medium. *In vitro* biofilms, as employed in the present study, are perfused with fresh medium. Whilst the rate of removal of extracellular products can be readily determined by assay of the spent medium, dilution effects within the body of the biofilm cannot be calculated without knowledge of the biofilm volume. Steady-state levels of extracellular products within the spent medium are not therefore equivalent. In order to make direct comparisons between the two modes of growth in the present study the units for each were expressed as productivities (units per 10^8 cells h⁻¹) relative to the resident population in the chemostat and within the biofilm, respectively.

Extracellular protease production (Fig. 1) increased significantly with increases in growth rate for the biofilm cultures and in all cases was greater than for chemostat populations growing at the same growth rate. Protease production by the planktonic chemostat cultures was maximal at intermediate growth rates (about 0.15–0.25 h⁻¹) and in no instance exceeded the productivity of biofilms.

The medium used in these studies contained no added iron and utilized Analar-grade reagents (BDH). Whilst control of growth rate was through limitation of the availability of carbon substrate, iron availability was sufficiently low to cause the expression of an iron-deprived phenotype. Iron chelators, presumed to be siderophores, were therefore produced during the growth of these cultures. The production of iron chelators by these biofilm cultures increased with growth rate (Fig. 2) and was significantly greater than for planktonic populations of cells. In contrast, iron chelation in chemostat cultures was greater at the slower growth rates. Such productivity was at a constant level of about 1.6 units per 10^8 cells h⁻¹ at growth rates of between 0.02 and 0.1 h⁻¹ and about 0.02 units per 10^8 cells h⁻¹ at faster growth rates. Similar dependence of siderophore production on growth rate has been reported for *Klebsiella pneumoniae* (Lodge *et al.*, 1986), when, under various levels of iron availability in continuous culture, siderophore levels and also expression of a 39 kDa peptidoglycan-associated protein increased with growth rate. The iron-chelation assay employed is not necessarily indicative of the production of siderophores, giving only a measure of the total iron-binding capacity of the culture supernatant. Whilst the results might therefore imply an induction of a high-affinity uptake mechanism for iron-associated siderophores at the faster growth rates, where presumably iron requirements by the cells are greatest, they might also indicate the production of weak iron chelators such as phosphate or organic acids. If it is assumed that the iron chelation comes from the production of siderophores then leaching from the biofilm cultures will dramatically reduce their effectiveness as an iron-uptake system. Greatly enhanced productivities of siderophores at the faster growth rates, when exopolymer deposition around the cells is reduced, might be as a response to severe iron deprivation.

Fig. 2. Effect of specific growth rate upon the production of siderophores by adherent biofilms (▲) and planktonic populations (■) of *S. epidermidis* grown in a peptone-supplemented simple-salts medium containing no added iron.

Iron chelator and protease production do not appear from the above data to be co-induced either in biofilms or planktonic culture. Such might be the case if a function of proteases were to liberate iron from naturally occurring iron carriers such as transferrin and lactoferrin (Griffiths, 1983).

Giwerzman *et al.* (1991) reported that biofilms of *Pseudomonas aeruginosa* were less susceptible to β -lactam-induced production of β -lactamases than were planktonic populations. They attributed this, at least in part, to reduced growth rates of the biofilm relative to the planktonic cells, and in part to trapping of the β -lactamase within the glycocalyx. In the present study it would appear that not only do attached populations have greater productivity of proteases than planktonic cells but that this productivity increases as exopolymers become less abundant within the biofilm and presumably entrapment of the enzyme decreases.

Effect of specific growth rate on EPS production

Levels of EPS associated with the planktonic cells and with the biofilms were expressed, relative to dextran, as μg per 10^8 cells (Fig. 3). At specific growth rates approaching μ_{max} the levels of EPS associated with planktonic and biofilm populations were similar. At slower rates of growth, however, EPS levels were markedly greater for the attached populations, in spite of growth under carbon-limitation. In *P. aeruginosa*, expression of a mucoid phenotype has been shown to be under the control of an environmentally responsive class of genes (Deretic *et al.*, 1989). Similar classes of genes are

Fig. 3. Effect of specific growth rate upon the production of EPS by adherent biofilms (▲) and planktonic populations (■) of *S. epidermidis* grown in a peptone-supplemented simple-salts medium containing no added iron.

probably associated with EPS production in staphylococci. Indeed, levels of EPS associated with the biofilm cells were inversely related to their specific growth rate/medium perfusion rate. Analysis of collected cells which had dispersed spontaneously from the biofilms showed them to have similar levels of EPS associated with them as the chemostat-derived samples. Such results are compatible with the notions that attachment induces EPS production at rates which are inversely proportional to growth rate and/or that EPS is preferentially retained by the attached cells on division and release of a daughter cell to the culture eluate.

Conclusions

The evidence presented supports the view that attachment of *S. epidermidis* cells to surfaces and their formation into biofilm is associated with marked alterations in the production of extracellular virulence factors. Such changes will substantially alter the phenotype expressed *in vivo* following growth in association with indwelling devices.

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