Flow cytometry and other techniques show that *Staphylococcus aureus* undergoes significant physiological changes in the early stages of surface-attached culture

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The techniques of flow cytometry, scanning and transmission electron microscopy, and confocal scanning laser microscopy were used to study the physiology of *Staphylococcus aureus* in the early stages of surface-attached culture, and to make direct comparisons with planktonic bacteria grown under the same conditions. Attached bacteria growing in nutrient-rich batch culture were found to go through the same growth phases as equivalent planktonic cultures, but with an exponential growth rate of about half that of the planktonic bacteria. Viability of attached bacteria was very high (around 100%) throughout the first 24 h of growth. The size and protein content of attached bacteria varied with growth phase, and both measurements were always smaller than in planktonic bacteria at equivalent growth phases. Respiratory activity per bacterium, as measured by flow cytofluorimetry, and corrected for cell volume, peaked very early in attached cultures (before the first cell division) and declined from then on, whereas in planktonic bacteria it peaked in late exponential phase. Attached and planktonic bacteria showed thicker cell walls in stationary phase than in exponential phase. Membrane potentials of planktonic and attached bacteria were similar in stationary phase, but were much lower in exponential-phase attached cells than in the equivalent planktonic cells. It is apparent that a range of significant physiological adaptations occur during the early phases of attached growth.

**Keywords:** *Staphylococcus aureus*, flow cytometry, attachment, biofilm

INTRODUCTION

In natural habitats most bacteria are found adhered to surfaces, either as individuals or as components of a biofilm (Marshall, 1976), and the great majority of problems caused by microbial growth (e.g. in industrial situations, disease states, or the colonization of implants in human patients) involve bacteria in an adhered condition. Studies of the growth of adhered bacteria (or other microbes) have mostly concentrated on the problems arising from such growth and on the means for its control or prevention, though there have also been numerous studies of the effects of adhesion on the genetic responses of the microbes (Goodman & Marshall, 1995). Whether the process of attachment to a surface itself directly triggers physiological change has not been addressed quantitatively. Thus, the most well-known property of adhered bacteria is their increased resistance to biocides (Costerton *et al.*, 1987), but the extent to which this results from changes in bacterial physiology rather than a capacity of the polymer matrix of the biofilm to limit the rate of antibiotic diffusion is

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Abbreviations: CLSM, confocal laser scanning microscopy; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; DIBAC(3), bis-1,3-dibutylbarbituric acid trimethine oxonol; FALS, forward angle light scatter; FITC, fluorescein isothiocyanate; SEM, scanning electron microscopy; TEM, transmission electron microscopy.
not fully resolved (Nichols et al., 1989; Anwar et al., 1992). Similarly, van Loosdrecht et al. (1990) list a range of diverse physiological changes (including growth yield and growth rate) observed to be associated with adherence to a surface, but conclude that there is no compelling evidence that any such changes are directly influenced by adhesion, and may instead result from a modification of the cells' surroundings by the surface. Other consequences of attachment to susceptible host tissue surfaces include the up-regulation of genes (e.g. alginate production; Davies & Geesey, 1995) and the production of specific proteins (Dagostino et al., 1991; Möller et al., 1998; O'Toole & Kolter, 1998). A study of morphological changes and surface colonization behaviour in Acinetobacter sp. (James et al., 1995) is one example of the use of digital imaging to study phenotypic changes consequent on attachment.

Whether or not they are the direct or indirect results of attachment, it is clearly important that the physiological adaptations that accompany the attachment of bacteria to surfaces should be more clearly elucidated. It is likely that the direct effects of membrane attachment on bacteria will be distinct from effects that arise from their incorporation into an extensive biofilm structure. Consequently, if the specific effects of attachment are to be elucidated, it is essential that attached cultures should be examined at an early stage in their development when bacterial densities are still low. This fact has recently been emphasized by the results of Williams et al. (1997) who showed that although young cultures of Staphylococcus aureus attached to silicone surfaces showed phenotypic resistance to some antibiotics, the level of resistance was much lower than is usually associated with the bacteria of mature biofilms.

An essential analytical requirement in the study of attached cultures is that measurements are made either in situ (i.e. when the bacteria are still attached) or very rapidly following their removal from the surface on which they are growing. In this context, flow cytometry offers a very promising technique for rapid analysis of large numbers of bacteria, permitting measurement of the size and macromolecular content of individual cells at a rate of about 1000 cells s⁻¹ (Boye & Løbner-Olesen, 1990). Thus it has been used to determine, for example, physiological differences between exponential-phase and stationary-phase cells of Escherichia coli and other bacteria (Paauw et al., 1977), cell size and cellular DNA and protein content of E. coli cultures (Boye & Steen, 1993), and antibiotic sensitivity of S. aureus (Suller & Lloyd, 1998).

In the present paper we use flow cytometry, in combination with confocal laser scanning microscopy (CLSM) and electron microscopy, to examine some physiological properties of the important human pathogen S. aureus during the early hours of growth on silicone surfaces. To the best of our knowledge, this is the first paper to report the use of flow cytometry in the investigation of attached bacterial cultures.
added to 1 ml MHB containing suspended bacteria or a catheter disc with attached bacteria and allowed to stain for 5 min at room temperature. Stained organisms were analysed by flow cytometry for fluorescence (490–520 nm).

**Staining with FITC.** Total protein content of individual bacteria was determined by measuring fluorescence after staining with FITC. This is a widely adopted procedure, validated for bacteria (Allman et al., 1990) and eukaryotes (Lloyd, 1993). Ethanol-fixed bacteria were centrifuged to remove fixative, and washed and resuspended in ice-cold Tris/HCl buffer (10 mM, pH 7.4). FITC solution (20 μl, 1 mg ml⁻¹ in ethanol) was added to give a final concentration of 20 μg ml⁻¹ and allowed to stain at room temperature for 30 min. The organisms were then centrifuged, resuspended and washed in Tris/HCl, and analysed by flow cytometry for forward angle light scatter (FALS) (as a measure of size) and fluorescence (490–530 nm) for total protein.

**Staining with CTC.** This dye is reduced by flavoprotein components of the prokaryotic respiratory chain (Smith & McFeeters, 1996) and is commonly used as an indicator of respiratory activity (Rodriguez et al., 1992; Schaeule et al., 1993). CTC solution (20 mg ml⁻¹ in water) was added to growing cultures of attached or planktonic cells to give a concentration of 600 μg ml⁻¹ and staining allowed to occur in darkness for 20 min. The organisms were then centrifuged, resuspended and washed in Tris/HCl, and analysed by flow cytometry for fluorescence (490–520 nm).

**Staining with rhodamine 123.** Rhodamine 123 is a voltage-sensitive cationic dye that is electrophoretically taken up into energized bacteria by virtue of the trans-membrane electrochemical potential (negative inside) of the plasma membrane (Mason et al., 1993). Dye (20 mg ml⁻¹ in water) was added to growing cultures to give a concentration of 0.2 mg ml⁻¹, and staining allowed to occur in darkness for 30 min. Samples were analysed immediately using CLSM.

**CLSM.** Planktonic bacteria or silicone discs with attached bacteria were mounted on glass slides with addition of carboxymethylcellulose (5%, w/v, for attached cultures; 15%, w/v for planktonic cultures) to increase viscosity, and 2% 1,4-diazabicyclo[2.2.2]octane (DABCO) antifade as a scavenger of oxygen radicals. The instrument employed was a Molecular Dynamics Sarastro confocal scanning microscope. Specimens were scanned using a 25 mW argon laser with appropriate excitation and emission filters for rhodamine (514/563 nm). To reduce photobleaching of fluorescence, the laser output was set at 15 mW and attenuated using a 30% neutral density filter. Specimens were examined using oil immersion objectives, magnification ×60 (50 μm confocal aperture) or ×100 (100 μm confocal aperture). Series of optical sections (512×512 pixels; 0.5 μm thick) were taken through entire organisms at a spacing of 0.7–1.0 μm. Three-dimensional projections were prepared from the section series using Molecular Dynamics ‘Imagespace’ volume-rendering software running in Silicon Graphics UNIX workstations. Stereo projections were prepared either as stereo pairs or two-colour overlays (anaglyphs: examined using red/green-filtered spectacles) and printed using a Shinko CHC-S446i dye sublimation colour printer.

**SEM.** Attached or planktonic bacteria were fixed in glutaraldehyde solution (3%, v/v, pH 7.4) for 1 h and transferred to phosphate buffer (pH 7.4) for 24 h. Attached cultures were then sonicated to release bacteria. Buffer-suspended bacteria (5 ml volumes) were passed through 0.1 μm membrane filters and the filters with retained bacteria were dehydrated through 50, 70 and 100% (v/v) ethanol (15 min at each ethanol concentration) and critical-point-dried at 1200 bar (120 kPa) pressure at 40 °C using liquid CO₂ (Balzers 030 critical point drier). Filters were mounted on aluminium stubs, gold-sputter-coated (Edwards S150B sputter coater) and viewed on a Hitachi field emission scanning electron microscope. Cell diameters were determined by manual measurement of longest diameters from enlarged photographic prints. Mean diameters were calculated from three samples of 50 cells selected randomly from triplicate culture samples.

**Transmission electron microscopy (TEM).** Attached or planktonic bacteria were fixed as described for SEM. Attached cultures were then sonicated in sodium cacodylate buffer (pH 7.4) to release bacteria. Bacterial suspensions (from either type of culture) were then centrifuged at 2000 r.p.m. for 5 min and resuspended in 1% Millonig’s phosphate-buffered osmium tetroxide for 1.5 h at 4 °C. After centrifugation, bacteria were resuspended in 200 μl bovine serum albumin (4% in Tris/HCl, 50 mM, pH 7.4) and 20 μl glutaraldehyde (25%, v/v, in Tris/HCl pH 7.4) was added. The suspension was immediately centrifuged at 2500 r.p.m. for 5 min. The pellet was removed and cut into cubes of volume approximately 1–2 mm³. Cubes were dehydrated as for SEM, placed in Lemix (EMSscope Laboratories)/ethanol (50:50, v/v) for 1 h, transferred to 100% fresh Lemix, incubated for 1 h at room temperature on a rotator, transferred again to fresh 100% Lemix and incubated on a rotator overnight at room temperature. Samples were then embedded in 100% Lemix in BEEM capsules at 60 °C for 18 h. Sections (60 nm thickness) were cut on an LKB Ultratome III, picked up on copper grids, stained for 1.5 h with uranyl acetate alcoholic solution and for 30 min with lead citrate, and viewed on a Hitachi transmission electron microscope.

**RESULTS**

**Growth characteristics of suspended and attached cultures**

Planktonic cultures were inoculated with a suspension of approximately 1 × 10⁹ stationary-phase bacteria and Fig. 1 shows a typical example of the growth curves obtained. Exponential growth phase occurred up to about 2 h with a mean generation time of 27 ± 2.1 min.

Attached cultures were grown using the method of Williams et al. (1997). Cultures were inoculated with approximately 1 × 10⁹ viable stationary-phase bacteria previously attached to silicone discs. Growth was followed by sonically removing the bacteria and enumerating them by viable counts on nutrient medium. Fig. 1 shows that the attached cultures followed conventional growth kinetics; the number of viable bacteria increased exponentially for the first 4 h of incubation, after which growth slowed and reached stationary phase by 8 h. During incubation, the broth medium surrounding the discs became turbid owing to the growth of detached bacteria. These followed a growth curve similar to that of suspended cultures but some 2–3 h delayed (Fig. 1). The timings of growth phases for attached and detached cells within the same culture vessel were therefore similar, with exponential phase ending at about 4 h. During exponential growth the
mean generation time for attached cells was $57 \pm 4.9$ min.

Williams et al. (1997) have shown that in this system, attached cultures initially comprise single well-spaced bacteria; at stationary phase, many single cells remain but most bacteria are present in aggregations that vary in size from 2 to about 100 cells. These characteristics were also typical of attached cultures used in the present investigation, and TEM showed that attached bacterial cells sampled after 5 h incubation were at various stages of growth and division, indicative of an actively growing culture, whereas those from 24 h samples showed no evidence of cell division (not shown).

**Estimation of the viability of attached bacteria using DiBAC$_3$(3)**

The cytofluorograph distributions in Fig. 2 compare dye uptake by exponential-phase planktonic bacteria (live and heat-killed) and attached bacteria. The live cultures of both planktonic and attached bacteria show very little uptake of the fluorescent dye, whereas the heat-killed bacteria all show significant uptake, the larger cells taking up proportionately more than the smaller ones. Essentially identical results were obtained with attached cultures of different ages (up to 24 h), and with bacteria that had been sonically removed from the membrane prior to staining. It is apparent therefore that attached cultures have very high viability levels and that this is not diminished by the sonication procedure used for their removal.

**Bacterial size**

Sizes of individual bacteria were estimated cytofluorimetrically by measuring FALS; this parameter measures light scattered at a forward angle of less than $15^\circ$ using a dark field configuration (Steen et al., 1989). The measurement is a complex function of size and refractive index, but for particles of bacterial size is closely correlated with volume (Allman et al., 1990). Median cell size was clearly related to the growth stage of the culture (Fig. 3). In suspended cultures, median cell size initially increased rapidly, reaching a peak in late exponential phase (at about 2 h) and declining again to a minimum at 12 h. Sizes of attached bacteria also showed a pattern of increase followed by decline. In common with suspended bacteria, median cell size peaked around late exponential phase, in this case at 5 h. The peak median cell size of suspended bacteria was
Physiology of attached *S. aureus* cultures

about 37% greater than that for attached bacteria. FALS measurements can be made on stained or unstained bacteria. Fig. 3 shows the results obtained with unstained bacteria, and essentially identical results were obtained with FITC-stained bacteria. Use of mithramycin/ethidium bromide-stained bacteria produced peaks at the same times as above, but with sharper profiles caused by a more rapid decrease in the FALS signal following the maxima (not shown).

**Total protein content**

The variation in median protein content is shown in Fig. 4. The pattern of variation was similar to that observed for cell size, with maxima occurring at 2 h in planktonic cell cultures and at 5 h in attached cultures. Plots of cell size versus protein content showed that the two parameters vary proportionally and, therefore, that cytofluorimetric determination of total protein per cell can be used as an indication of cell size.

**Respiratory activity**

This was measured cytofluorimetrically after staining bacteria with CTC. Plots of median cell fluorescence versus time again produced peaks at 2 h for planktonic cultures and 5 h for attached cultures, showing that the largest bacteria possess the greatest total respiratory activity (not shown). Plots of CTC fluorescence versus cell size showed that specific respiratory activity (fluorescence per unit volume) of planktonic bacteria peaks in late exponential phase, but in attached bacteria respiration rises to a maximum early in exponential phase and tails away thereafter (Fig. 5). At most stages of growth, specific respiratory activity in attached bacteria was considerably higher than in planktonic bacteria.

**Measurement of cell diameters by SEM**

As cytofluorimetric measurements had indicated that sizes of suspended and attached bacteria peaked in late
Table 1. Cell sizes of *S. aureus* in planktonic and attached culture

For each culture condition, samples of 50 bacteria were chosen randomly from each of 3 independent photographs; 99% confidence intervals are shown.

<table>
<thead>
<tr>
<th>Culture type</th>
<th>Growth phase</th>
<th>Mean cell diameter (μm)</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planktonic</td>
<td>Exponential (2 h)</td>
<td>1.01 ± 0.03</td>
<td>0.127</td>
</tr>
<tr>
<td>Attached</td>
<td>Exponential (5 h)</td>
<td>0.78 ± 0.03</td>
<td>0.179</td>
</tr>
<tr>
<td>Planktonic</td>
<td>Stationary (24 h)</td>
<td>0.75 ± 0.014</td>
<td>0.053</td>
</tr>
<tr>
<td>Attached</td>
<td>Stationary (24 h)</td>
<td>0.69 ± 0.012</td>
<td>0.087</td>
</tr>
</tbody>
</table>

For each culture condition, samples of 50 bacteria were chosen randomly from each of 3 independent photographs; 99% confidence intervals are shown.

Table 2. Cell wall thicknesses of *S. aureus* in planktonic and attached culture

For each culture condition, samples of 50 bacteria were chosen randomly from each of 3 independent photographs; 99% confidence intervals are shown.

<table>
<thead>
<tr>
<th>Culture type</th>
<th>Growth phase</th>
<th>Mean cell wall thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planktonic</td>
<td>Exponential (2 h)</td>
<td>14.0 ± 0.86</td>
</tr>
<tr>
<td>Attached</td>
<td>Exponential (5 h)</td>
<td>12.8 ± 1.2</td>
</tr>
<tr>
<td>Planktonic</td>
<td>Stationary (24 h)</td>
<td>35.7 ± 2.2</td>
</tr>
<tr>
<td>Attached</td>
<td>Stationary (24 h)</td>
<td>22.2 ± 0.10</td>
</tr>
</tbody>
</table>

Exponential phase (at 2 and 5 h, respectively), samples from these growth points were examined by SEM and their sizes compared with those of stationary-phase cells (24 h). Mean cell diameters are shown in Table 1. The data indicate clearly that exponential-phase bacteria, planktonic or attached, are significantly larger than their stationary-phase counterparts. Planktonic bacteria are also significantly larger than attached bacteria at the exponential phase (at 2 and 5 h, respectively).

Fig. 6. Membrane potentials of *S. aureus*. Bacteria were stained with rhodamine 123 and examined by CLSM. Photographs show exponential-phase planktonic (a), stationary-phase planktonic (b), exponential-phase attached (c) and stationary-phase attached (d) bacteria. White bar, 1 μm.
equivalent growth phase (exponential or stationary). In each of the above cases, statistical analysis shows that the difference between means greatly exceeds the minimum significant difference (0.022). Cytofluorimetric measurements are therefore supported by microscopic measurements.

The range of variation in diameters provides confirmatory evidence for the growth status of the bacteria. In both samples from exponentially growing bacteria the coefficients of variation seen within samples (planktonic = 0.127; attached = 0.179) are consistent with a volume range of at least twofold within each sample (volume being proportional to the cube of the diameter). This would be expected of actively dividing cultures where new daughter cells will be about half the size of cells that are about to divide. Size variation in stationary-phase cultures (planktonic or attached) was only about half of that observed in exponential cultures.

Measurement of cell wall thickness by TEM

TEM was used to measure and compare cell wall thicknesses of planktonic and attached cultures in both exponential and stationary phase (Table 2). Bacteria in stationary phase had much thicker cell walls than those in exponential phase; in planktonic cultures the increase in thickness was about 2.5-fold and in attached cultures about 1.7-fold. Cell walls of stationary-phase planktonic bacteria were about 1.6 times thicker than those of stationary-phase attached bacteria. In all these cases the differences were statistically significant with the differences between means greatly exceeding the minimum significant difference (1.33). Bacteria from the two exponential cultures had cell walls of similar thickness; planktonic bacteria had the higher mean value, but not significantly so.

CLSM

This was carried out on planktonic and attached cultures after staining with rhodamine 123. This dye requires a membrane potential for transport into the cell and is taken up in an energy-dependent manner (i.e. uptake is sensitive to uncoupling agents) (Mason et al., 1993). Amount of dye taken up is therefore directly related to the level of energy metabolism in the cell.

In large fields of bacteria examined, populations of exponential-phase planktonic bacteria had taken up more dye than exponential-phase attached bacteria. Thus the distribution of voltage-sensitive dye between the environment and the intracellular space was clearly different in the two population types. At higher magnification it was possible to quantify this distribution (Fig. 6). In the examples shown, which are typical examples from four independent experiments, the bacteria analysed (outlined in red) had measured peak pixel intensities of 255 and 140, respectively. Stationary-phase bacteria took up very little dye and typical examples of both planktonic and attached cells gave peak pixel intensities of about 65.

DISCUSSION

S aureus was grown on silicone surfaces using the system described by Williams et al. (1997). The attached bacteria were shown to follow the pattern of growth normally seen in liquid batch cultures, comprising a period of exponential increase followed by a gradual cessation of division and entry into stationary phase. The timing of the transition from exponential to stationary phase paralleled that of planktonic bacteria present in the same culture, which indicates that both attached and planktonic populations are similarly affected by exhaustion of nutrients and build up of any inhibitory products. The degree of variation in bacterial size seen in scanning electron micrographs of attached exponential-phase cells was consistent with the existence of an actively growing and dividing population, whereas the greater uniformity of size in stationary-phase samples supported the view that these were non-dividing populations.

Staining of attached bacteria with DiBAC$_4$(3) followed by cytofluorimetric analysis indicated that most, possibly all, attached bacteria were viable, even after incubation for 24 h, when they had been in stationary phase for some 16 h. This contrasts with the situation in mature biofilms where a high proportion of cells may be non-viable (Kinniment, 1994; Kinniment & Wimpenny, 1992).

Analysis of FALS during cytometry showed that attached bacteria increased in size during the exponential growth phase and reached maximum size at about 5 h, which coincided with the end of exponential phase. Size decreased again as bacteria entered stationary phase. Bacteria in independent planktonic cultures were considerably larger but showed a similar pattern of size variation. These cultures reached the end of exponential phase at 2–3 h and the maximum bacterial size also occurred at this time. The same patterns of size variation (in attached and planktonic cultures) were indicated by cytofluorimetric measurements of total protein content per cell, and also by direct measurements of cell diameter in scanning electron micrographs. It has long been established that cell size and macromolecule (DNA, RNA, protein) content in planktonic bacterial cultures are directly related to growth rate (Schaechter et al., 1958); our experiments confirm that the same principles apply to attached cultures.

Cytofluorimetric analysis of CTC-stained bacteria indicated that respiratory activity per individual cell also peaked in late exponential phase for both attached and planktonic cultures. However, expression of respiratory activity per unit cell volume indicates that for attached bacteria specific respiratory activity is at a maximum at a very early stage of growth. Williams et al. (1997) have shown that in the attached culture system used here, bacteria are initially present mostly as single, well-spaced cells. Considering that the attached bacteria have an initial lag phase, and that in the succeeding exponential phase they have a doubling time of 57 min, the observed peak in specific respiratory activity at about
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1 h must have occurred when bacteria were still in the singlet stage, and appears to represent the operation of a regulatory event that is triggered very soon after the start of attached growth. Induction of the alginate genes during the establishment of attached cultures of *Pseudomonas aeruginosa* was up-regulated after 15 min (Davies & Geesey, 1995).

There have been numerous reports that have compared aspects of physiology and gene expression in planktonic and surface-attached bacterial cultures. For example, growth on a solid surface produced a smaller cell size in *E. coli* (Lorian, 1986) and thicker cell walls in staphylococci (Lorian et al., 1985); other bacteria have been shown to produce β-haemolysin (Lorian & Popoola, 1972) or luminescence (Nealson & Hastings, 1979) only when growing on solid surfaces; use of reporter gene technology has demonstrated that surface-attached growth results in switch-on of some genes and switch-off of others (Dagostino et al., 1991; Moller et al., 1998; Davies & Geesey, 1995). In contrast to these, our studies have followed physiological changes that occur during the establishment of attached bacterial cultures, and have made comparisons with bacteria at the equivalent stages of planktonic culture. The importance of this approach is particularly apparent in our measurements of cell wall thickness, where the cell wall of planktonic bacteria may be thicker (in stationary phase) or thinner (in exponential phase) than that of stationary-phase attached bacteria.

The studies described here have dealt with the broad physiological parameters of viability, cell size, and total protein content and respiratory activity of individual cells, and have demonstrated that flow cyt fluorimetry has considerable potential for measurement of the properties of bacteria in attached culture. Cyt fluorimetry with more specific fluorescent probes, for example specific mRNA probes, could be used to follow changes in gene expression patterns that are consequent on attachment to surfaces. Fluorescently labelled 16S rRNA probes have already been used in conjunction with flow cyt fluorimetry to identify bacterial species in biofilms (e.g. Wallner et al., 1993).

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