Contribution of culture media and chemical properties of polystyrene tissue culture plates to biofilm development by Staphylococcus aureus

Staphylococcal biofilm development on implanted biomaterials represents an important virulence determinant in the pathogenesis of device-related infections. The prevailing environmental milieu and growth media supplements such as NaCl, ethanol, glucose and sub-inhibitory concentrations of antibiotics strongly influence biofilm development. In the laboratory, the microtitre plate assay is one of the commonest methods used to measure the biofilm-forming capability of Staphylococcus aureus isolates. This semi-quantitative measurement of biofilm formation involves growing bacterial cultures in individual wells of 96-well polystyrene plates according to the method of Christensen et al. (1985). In our experiments bacteria were grown at 37°C for 24 h before being vigorously washed three times with distilled H2O and dried for 1 h at 56°C as recommended by Gelosia et al. (2001) prior to staining with a 0.4% crystal violet solution. The absorbance of adhered, stained cells was measured at 492 nm using a Multiskan plate reader (Flow Laboratories).

We have previously observed that the restriction-deficient laboratory strain RN4220, normally used as an intermediate cloning host, is capable of variable but significant levels of biofilm formation and that NaCl can further induce biofilm development in this strain. RN4220, which is a derivative of NCTC 8325-4, is known to harbour a small deletion in the rsbU gene and is therefore deficient in the stress responsive sigma factor, σB. In addition, these observations appear to contradict previous results, which demonstrated that in the absence of σB, biofilm formation could not be induced in this strain (Rachid et al., 2000). Significantly, Valle et al. (2003) reported that isolates of 8325-4 from different laboratories may have different biofilm forming capacities. To investigate this possibility further and to assess the σB status of our RN4220 strain we used PCR to verify the presence of the 11-bp deletion in the RN4220 rsbU gene as described previously by Kullik et al. (1998) (data not shown). In addition, we used RT-PCR to demonstrate that transcription of the σB-dependent genes asp23 (Gertz et al., 1999; Giachino et al., 2001) and csh9 (Gertz et al., 2000) were severely repressed in RN4220 and its parent strain 8325-4 compared to the rsbU-repaired derivative of 8325-4, SH1000 (Horsburgh et al., 2002) (data not shown). Therefore, given that our strain of RN4220 appeared to have the correct genotype we decided to examine whether differences in the culture media used or chemical properties of the polystyrene 96-well plates could explain the difference between our biofilm assay data and that of Rachid et al. (2000). The experiments of Rachid et al. (2000) were performed on cells grown in TSB medium, whereas in this study cells were grown in BHI broth. We therefore repeated our analysis of RN4220 using TSB-grown cells. This revealed that, consistent with the findings of Rachid et al. (2000), the biofilm-forming capacity of RN4220 was diminished when grown in TSB (Fig. 1), indicating that the choice of culture media strongly influences biofilm development in S. aureus. In addition, differences between the Oxoid TSB used in this study and the Difco TSB used by Rachid et al. (2000) may account for the weak but significant biofilm formed by RN4220 in the Oxoid TSB (Fig. 1). However, we still observed a strong induction of biofilm-forming capacity when RN4220 was grown in TSB supplemented with 3% NaCl (Fig. 1).

We examined next whether the chemical composition of the polystyrene 96-well tissue culture plates influences biofilm development. Throughout this study we used Nunc tissue-culture-treated plates, which have a hydrophilic, negatively charged surface. Untreated polystyrene is more hydrophobic. Therefore, we compared biofilm development by RN4220 cultures grown in BHI and TSB using Nunc tissue-culture-treated plates compared to untreated plates (Sterilin) (Fig. 1). This analysis revealed that RN4220 was incapable of biofilm formation on untreated 96-well plates, even when it was grown in media supplemented with NaCl. These observations may also contribute to the differences between our biofilm assays and those of Rachid et al. (2000), and suggest that the chemical composition of the polystyrene used in the manufacture of 96-well plates is a crucial variable in assays of biofilm formation by S. aureus. Moreover, tissue-culture-treated 96-well plates are optimized for eukaryotic cell attachment, which may also promote bacterial cell

Fig. 1. Comparative measurement of biofilm formation in S. aureus RN4220 grown in Oxoid BHI media and BHI supplemented with 4% NaCl (BHI NaCl) (a), or Oxoid TSB media and TSB supplemented with 4% NaCl (TSB NaCl) (b), using Nunc tissue-culture-treated 96-well plates or Bibby Sterilin untreated 96-well plates. The treated polystyrene plates have a negatively charged, hydrophilic surface compared to the untreated polystyrene, which is uncharged and more hydrophobic. Biofilm assays were performed at least three times and standard deviations are indicated.

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attachment. This in turn may suggest that lack of biofilm development by some \textit{S. aureus} strains on untreated polystyrene plates may sometimes be due to the absence of primary attachment rather than the inability to form biofilm.

Because RN4220 may harbour uncharacterized mutations (this strain is a derivative of 8325-4 and was chemically mutagenized to allow it accept foreign DNA), we repeated these experiments with 8325-4 and its rsbU-repaired derivative SH1000 (Horsburgh et al., 2002). Similar to the results obtained in RN4220, these experiments revealed that both 8325-4 and SH1000 were incapable of biofilm development on uncharged, hydrophobic polystyrene even when grown in the presence of NaCl or ethanol (Fig. 2). These data, together with the results of our analysis of RN4220 (Fig. 1), also suggest that rsbU is not required for biofilm induction by NaCl in \textit{S. aureus}.

Interestingly, these observations appear to contradict previous studies, which suggested that negatively charged materials, such as ionized plastics, Teflon or hydrophilic glass, are necessary for biofilm formation. This in turn may suggest that attachment of \textit{S. aureus} cells to different surfaces is complex. Apparently the influence of surface hydrophobicity and charge on staphylococcal adherence cannot be considered in isolation, but rather the combined contribution of these and other properties to the overall physical characteristics of a surface is clearly more significant.

In summary, we have identified two important variables in the microtitre plate biofilm assay, which is the most commonly used method for measuring staphylococcal biofilm formation. The physical properties of the polystyrene used in the construction of 96-well microtitre plates and, to a lesser extent, the commercial growth media used strongly influenced biofilm development by the \textit{S. aureus} strains 8325-4, RN4220 and SH1000. Our findings suggest that measurements of biofilm-forming capacity among \textit{S. aureus} isolates should be conducted with hydrophilic, negatively charged polystyrene and that \textit{S. aureus} is incapable of biofilm development on uncharged, hydrophobic polystyrene.

\textbf{Fig. 2.} Comparative measurement of biofilm formation in \textit{S. aureus} 8325-4 and SH1000 grown in Oxoid BHI media and BHI supplemented with 4% ethanol (EtOH) or 4% NaCl (NaCl) using Nunc tissue-culture-treated 96-well plates or Bibby Sterilin untreated 96-well plates. Biofilm assays were performed at least three times and standard deviations are indicated.

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