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Effects of carbon dioxide and sub-lethal levels of antibiotics on adherence of coagulase-negative staphylococci to polystyrene and silicone rubber

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Fifty coagulase-negative staphylococci (CNS) strains were investigated for adherence to both silicone rubber and polystyrene using a microtitre tray system. Culture in an atmosphere containing a physiological level of carbon dioxide (5% CO_2) profoundly affected adherent growth to both surfaces. Most strains adhered less well in this atmosphere compared to in air alone, with mean reductions in adherence of 84% and 86% to silicone rubber and polystyrene respectively. Occasional strains adhered better in 5% CO_2. The effects of antibiotic concentrations equivalent to 1/4 MIC of cefuroxime, ciprofloxacin, vancomycin and teicoplanin on the adherence of 10 CNS strains were also studied. Vancomycin and teicoplanin frequently increased adherence to silicone rubber and polystyrene compared to controls. The effects of antibiotics on adherence were not only strain dependent but also sometimes atmosphere or surface specific. Antibiotic-induced changes in adherence did not appear to correlate with changes in strain protein profiles or surface hydrophobicities.

Introduction

Bacterial adherence to host cells or foreign materials is an important factor in the pathogenesis of many infections. Interference with the mechanisms involved in adherence is seen, therefore, as an attractive way of either preventing or shortening infective episodes. The use of antimicrobial agents to modify bacterial adhesion *in vitro*, at concentrations that do not completely inhibit growth, is well recognized (Schifferli & Beachey, 1988*a*,*b*). However, relatively few studies have focused on the effects of sub-lethal concentrations of antibiotics on the adherence of bacteria, and in particular coagulase-negative staphylococci (CNS), to plastic surfaces (Schadow, Simpson & Christensen, 1987; Finch *et al.*, 1989). Given the importance of CNS as the major pathogens in medical device-associated infections, more information in this area is needed.

Using CNS isolates from patients on continuous ambulatory peritoneal dialysis (CAPD) with peritonitis, we have shown that culture in nutrient broth in an atmosphere containing physiological levels of carbon dioxide (CO_2) affects both bacterial cell surface chemistry and adherence potential (Denyer *et al.*, 1990). Physiological CO_2 tensions must be utilised to prevent marked precipitation in used peritoneal dialysis fluid, which in turn affects bacterial behaviour when this is employed as an alternative

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culture medium (Wilcox *et al.*, 1990). Recently, oxygen dependent exopolysaccharide production in *Pseudomonas aeruginosa* has been suggested to be important as a cause of treatment failure of left-sided endocarditis caused by this bacterium (Bayer *et al.*, 1989). This study investigated the effects of increased CO_2 levels on the adherence of CNS cultured in tryptone soya broth (TSB), with and without the presence of sub-lethal levels of antibiotics. We have also examined the relationships of atmosphere and antibiotic-induced changes in adherence, to the surface hydrophobicity and cell wall and membrane protein profiles of CNS strains.

Materials and methods

Bacterial isolates

A total of 50 CNS strains isolated from patients receiving CAPD and with a diagnosis of peritonitis, were chosen at random for initial study of the effects of CO_2 on bacterial adherence. Four other well-studied CNS from other sources were used for comparison (strains ATCC 35983/4—both *Staphylococcus epidermidis*; and strains ATCC 35981/2—both *S. hominis*) (Christensen, Baddour & Simpson, 1987; Schadow *et al.*, 1987). Ten of the 50 strains were selected to investigate the effects of sub-lethal concentrations of antibiotics on adherence. Strains were speciated using API Staph-Ident (API, Basingstoke, England) as follows (with the strain identification numbers of those studied further in parentheses: 38 strains of *S. epidermidis* (8, 31, 34, 49, 59, 112, 900, 902); five strains of *S. warneri/capitis* (79); three strains of *S. hominis*; two strains of *S. simulans*; one strain of *S. saprophyticus* (907); and one strain of *S. haemolyticus*.

Determination of minimum inhibitory concentrations (MICs)

The MICs of the following antibiotics for the ten CNS strains were performed in Tryptone Soya Broth (TSB-Oxoid Ltd, Hampshire, England), using a microtitre tray system and an initial inoculum of 10^6 cfu/ml: cefuroxime (Glaxo, Middlesex, England); ciprofloxacin (Bayer, Berkshire, England); vancomycin (Eli Lilly, Hampshire, England); and teicoplanin (Merrell Dow, Middlesex, England). MICs were performed in doubling dilutions in both air and air with 5% CO_2, and were read after incubation at 37°C for 18 h.

Adherence assay

Adherence to both polystyrene and silicone rubber was measured using a modified previously described method (Christensen *et al.*, 1985; Finch *et al.*, 1989). Polystyrene microtitre trays with flat-bottomed wells were used (M29A, Dynatech Laboratories Ltd, Sussex, England), half the wells in any one tray containing 5 mm diameter silicone rubber discs (Silicone Fabrications Ltd., Manchester, England) affixed with silicone grease (British Drug Houses, Dorset, England). CNS strains were cultured in TSB in quadruplicate wells with and without silicone discs, at 37°C for 18 h (initial inoculum 10^6 cfu/ml, and final well volume 250 μl). Following incubation, well contents were aspirated carefully and non-adherent organisms removed by washing three times with normal saline. Adherent bacteria were fixed in 25% v/v formaldehyde, stained with crystal violet and the plates then thoroughly washed to remove excess stain. After air-drying at room temperature, adherence to the bottom of wells was quantified by determining absorbance at 546 nm using an ELISA plate reader (SLT 210, Kontron Analytical Instruments, Zurich, Switzerland).

Adherence assays were performed in air and in air with 5% CO_2, with and without a ¼ MIC of cefuroxime, ciprofloxacin and vancomycin. In a separate group of experiments the effects of ¼ MIC vancomycin and teicoplanin were directly compared. Each experiment was performed at least three times and means were calculated. The adherence score for each strain was the mean A_{546} minus the A_{546} of an appropriate uninoculated control well. The % adherence was calculated by dividing the adherence score in the presence of antibiotic by that obtained in the absence of antibiotic. Thus a value of 100% is obtained if the antibiotic has no effect on adherence.

Cell wall and membrane protein analysis

Four CNS strains (numbers 8, 49, 59 and 902), selected for their range of antibiotic-induced adherence changes, were investigated for possible alterations in cell wall and membrane protein profiles. These strains were cultured, with and without a ¼ MIC of cefuroxime, ciprofloxacin and vancomycin, in 100 ml volumes of TSB in glass flasks, at 37°C for 18 h in air with 5% CO_2. Cells were harvested by centrifugation (10,000 *g* for 10 min) and then washed once in phosphate buffered saline (PBS—120 mM NaCl, 10 mM Na_2HPO_4, pH 7·4). Cell walls and membranes were separated by a modification of an existing method (Cheung & Fischetti, 1988). Briefly, cell wall digestion by lysostaphin was performed in a 30% w/v raffinose solution to stabilize resulting spheroplasts, which were then disrupted by sonication. Proteins were separated on 12·5% SDS-polyacrylamide gels and stained with Coomassie blue (Williams, Denyer & Finch, 1988).

Hydrophobicity measurements

The BATH technique (Rosenberg, Gutnick & Rosenberg, 1980) was used to measure the surface hydrophobicity in the 10 CNS strains studied in the adherence assay (cultured in air and in air with 5% CO_2). In addition strains 8, 49, 59 and 902 were also cultured in the presence of a concentration ¼ MIC of antibiotic (in air with 5% CO_2). Bacteria were washed and then suspended in a phosphate-urea-magnesium buffer (pH 7·1) to an A_{470} of 0·3. A 1·2 ml sample of this suspension was vortexed together with 0·2 ml of octane in a round-bottomed glass tube for 120 sec. The phases were allowed to separate at room temperature and the A_{470} of the aqueous phase was then measured. The reduction in A_{470} reflected the strain surface hydrophobicity. At least two different measurements were made in each instance and the mean calculated.

Results

The microtitre tray assay of adherent growth showed good reproducibility of results as described previously (Christensen *et al.*, 1985; Finch *et al.*, 1989). For example, the coefficient of variation for the adherence score of strain 907 to either silicone rubber or polystyrene was less than 10% (both within and between trays). A pronounced reduction in adherent growth to both silicone rubber and polystyrene was seen when bacteria were cultured in TSB in air with 5% CO_2, as compared to air alone. Viable

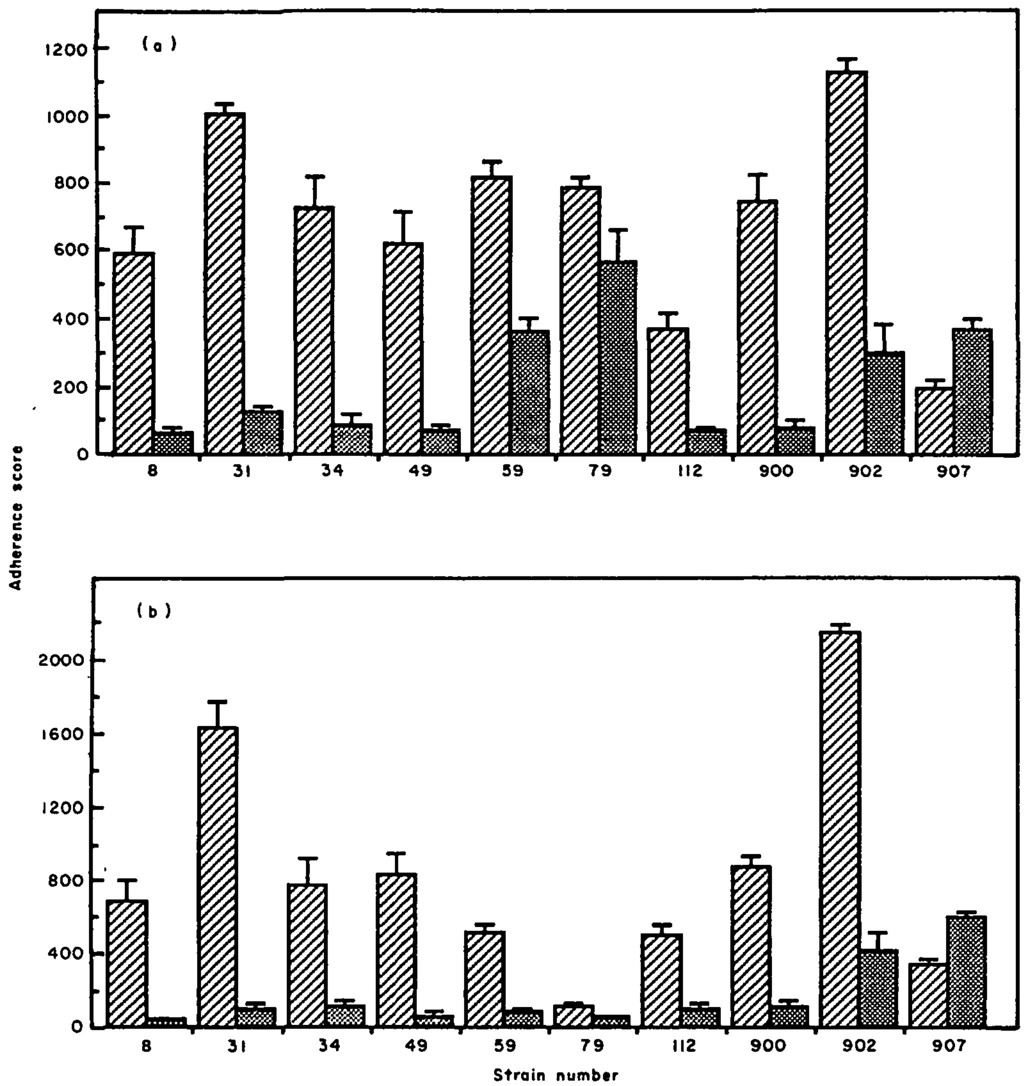


Figure 1. Effect of atmosphere (▨ air, ▩ 5% CO_2) on the adherence of coagulase-negative staphylococci to (a) silicone rubber and (b) polystyrene. Adherence score is the mean A_{546} minus the A_{546} of an uninoculated control well × 1,000. Errors bars represent SEM.

counting confirmed that the growth yield was similar in the two atmospheres. In general, strongly adherent CNS strains showed the largest reductions in adherence scores, but marked inter-strain variation was seen (Figure 1(a) and (b)). The mean reduction in adherence scores for the 50 CNS strains isolated from cases of peritonitis was 84% with silicone rubber and 86% with polystyrene. Similar mean decreases in adherence, of 70% and 80% respectively, were seen for those 10 CNS strains selected for further study. Only one strain (907) out of 50 consistently adhered more strongly in the CO_2 enriched atmosphere compared to air (89% and 76% increases to silicone rubber and polystyrene, respectively). There was a marked inter-strain variation in the relative adherence to silicone rubber and polystyrene (compare Figure 1(a) with 1(b)).

The pH of the culture of each strain was always lower (mean 0·24, S.D. 0·08 pH units) following incubation in air with 5% CO_2 (mean 5·93, range 5·71–6·01 units) compared to in air alone (mean 6·17, range 5·80–6·32 units).

The adherence in TSB of the four CNS strains from sources other than CAPD patients was also atmosphere-dependent. Strains ATCC 35983 and 35984 showed reduced adherence to both surfaces in air with 5% CO_2, similar to that seen above. However, strain ATCC 35981 adhered better to silicone rubber (36%) and polystyrene (296%) when grown in the CO_2 enriched atmosphere. Strain ATCC 35982 adhered poorly in both atmospheres but also showed increased adherence in air with 5% CO_2 (9% and 60% for silicone rubber and polystyrene, respectively). The adherence scores of the four ATCC strains was in order of decreasing scores: ATCC 35984 > ATCC 35983 > ATCC 35981 > ATCC 35982, as has been reported by others (Christensen *et al.*, 1985).

The combined effects of atmosphere and concentrations of cefuroxime, ciprofloxacin or vancomycin ¼ MIC of each antibiotic, on the adherence of 10 CNS strains, are shown in Figure 2(a) and 2(b). In general, cefuroxime commonly caused a reduction in adherence, whereas vancomycin frequently appeared to result in increased adherence. Ciprofloxacin caused changes midway between these two extremes, but rarely increased adherence. As adherence was nearly always much poorer in air with 5% CO_2, antibiotic-induced changes were less apparent when this incubation atmosphere was used. Changes in adherence caused by antibiotic exposure were sometimes atmosphere specific (e.g. ciprofloxacin-induced increased adherence of strain 902 in air with 5% CO_2) or surface specific (e.g. cefuroxime-induced reduced adherence of strain 907 to polystyrene).

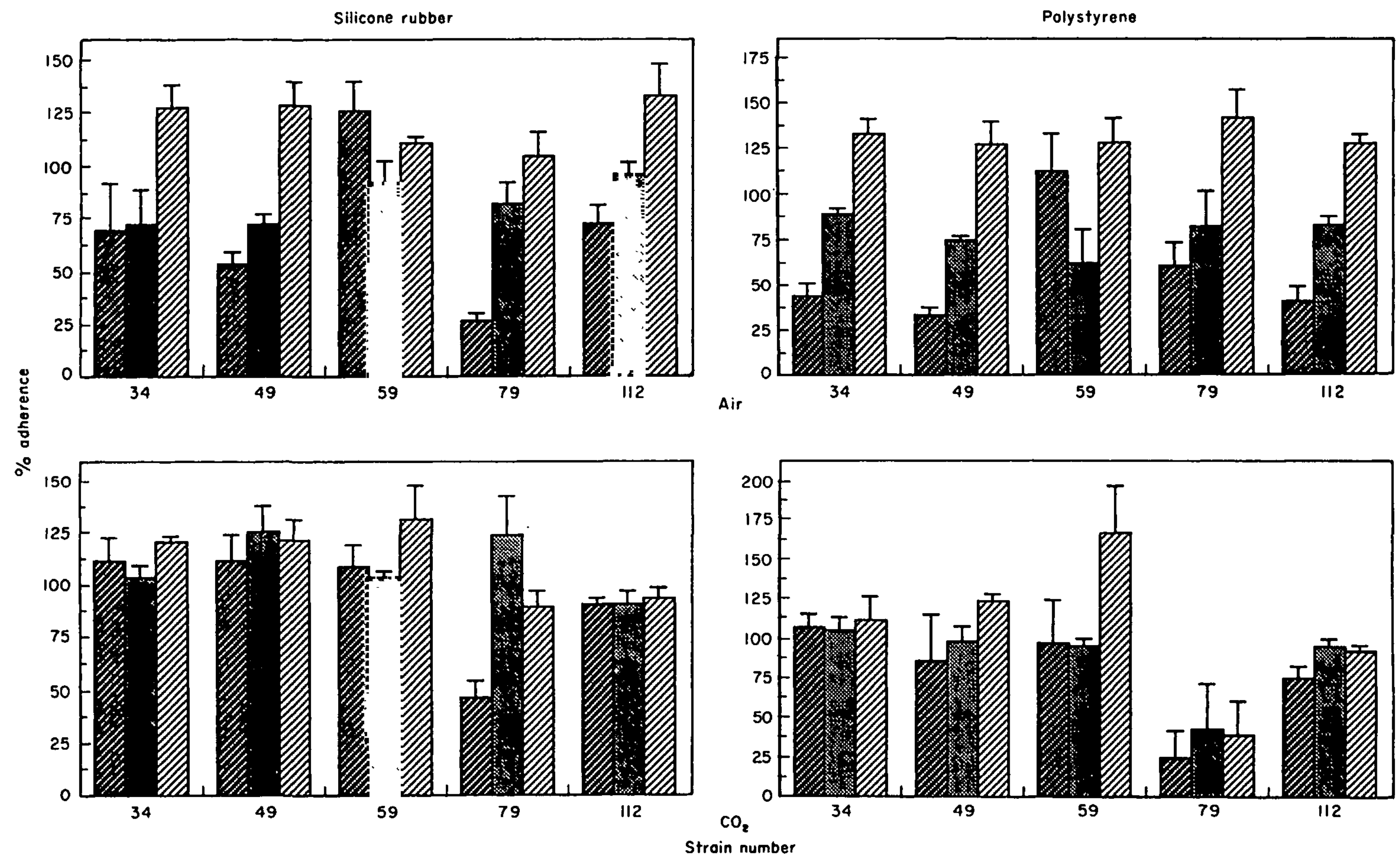
The effects of ¼ MIC of vancomycin and teicoplanin on adherence, to silicone and polystyrene (in both atmospheres) were very similar when compared directly (data not shown). For instance, the mean percentage increase in adherence score in antibiotic exposed strains compared to controls, was 22% with teicoplanin and 20% with vancomycin (expressed as the mean of changes in adherence to silicone rubber, in air with 5% CO_2, for 10 CNS strains).

The surface hydrophobicity of each of the above 10 CNS strains was very similar when culture had taken place in air compared to that in the CO_2 enriched atmosphere. The mean $\pm$ S.E.M. percentage of the initial A_{470} remaining in the aqueous phase for ten strains was as follows: 16 ± 8 (air) and 17 ± 8 (air with 5% CO_2). Similarly, there was no correlation between hydrophobicity measurements on strains 8, 49, 59 and 902 and antibiotic-induced changes in adherence.

The SDS-PAGE protein profiles of the cell walls and membranes of these same four antibiotic exposed strains (in air with 5% CO_2) showed only minor changes from controls. The one exception, however, was strain 902, in which two cell membrane proteins (29·5 and 30·5 kd) were apparently induced by exposure to ¼ MIC of cefuroxime (Figure 3).

Discussion

CNS are the predominant cause of medical device-associated infections (Archer, 1985). The ability of these organisms to adhere and grow on such foreign bodies is clearly important. We have demonstrated in this in-vitro study the dramatic effect on adherence


Silicone rubber
Polystyrene
% adherence
0
25
50
75
100
125
150
175
200
34
49
59
79
112
Air
CO$_2$
Strain number

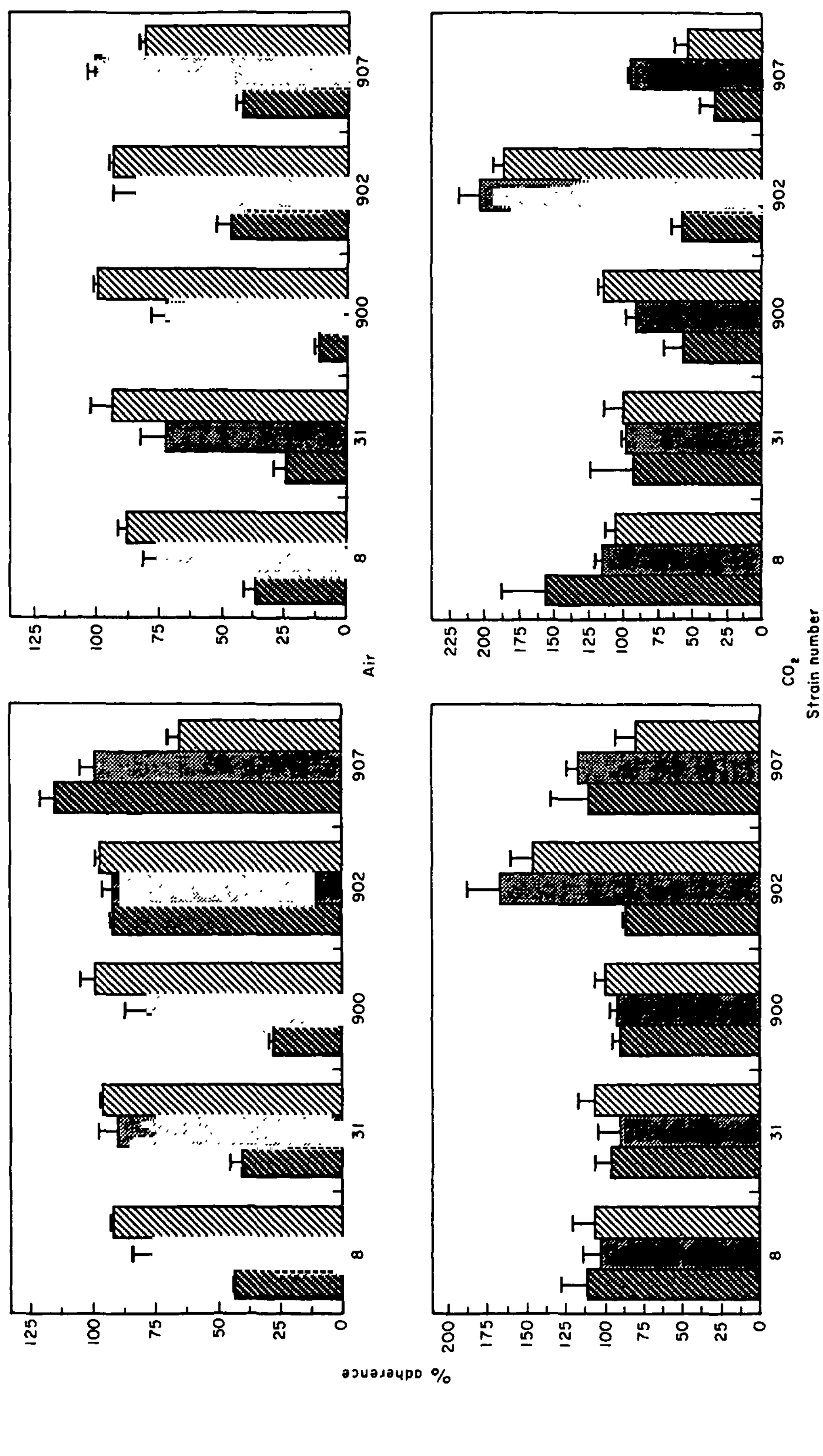


Figure 2. The effect ¼ MICs of antiobiotics (▨ cefuroxime, ▩ cirpofloxacin, ▨ vancomycin) on the adherence of 10 strains of CNS to silicone rubber and polystyrene, incubated in air and 5% CO_2; % adherence = $\frac{\text{adherence score in presence of antibiotic}}{\text{adherence score in absence of antiobiotic}}$. Error bars are SEM (minimum of three experiments).

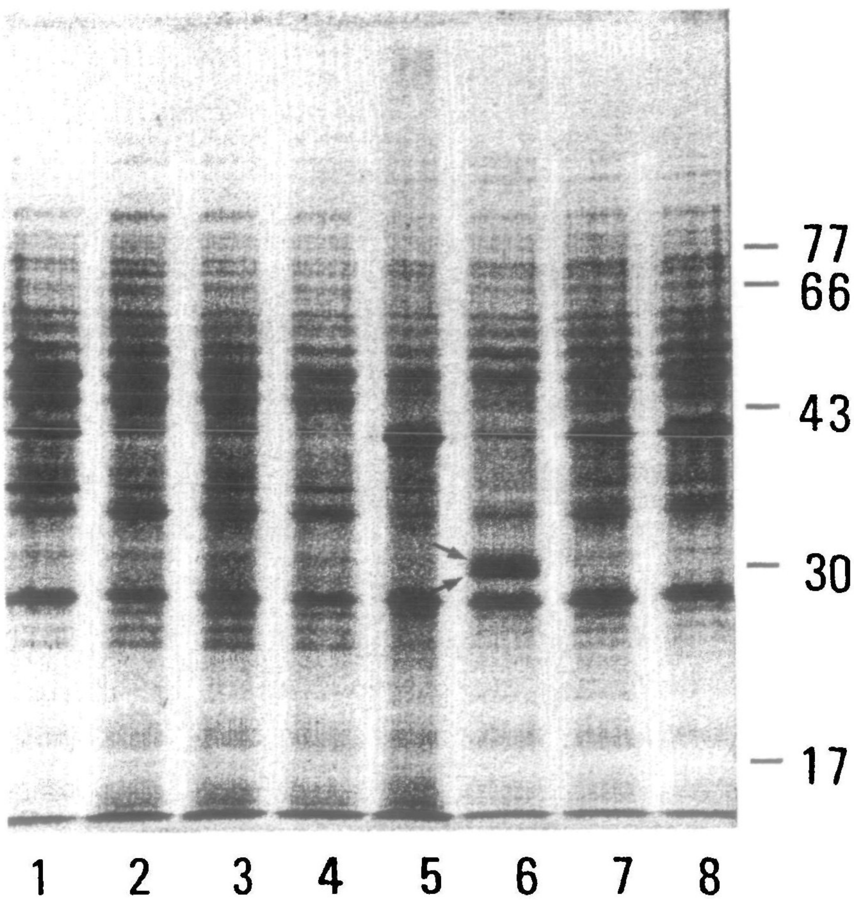


Figure 3. Cell membrane protein profiles of two CNS strains cultured with and without ½ MIC of antibiotics. Lanes 1–4, strain 8 and lanes 5–8, strain 902: lane 1: control; lane 2: culture with ½ MIC of cefuroxime; lane 3: culture with ½ MIC of ciprofloxacin; lane 4: culture with ½ MIC of vancomycin. Lanes 5–8 same order of antibiotic exposure as lanes 1–4. Molecular weights (KDa) are indicated on far right. Arrows indicate two membrane proteins apparently induced by exposure to cefuroxime in strain 902.

by CNS, of culture in air with 5% CO_2 as opposed to air alone. Employing this physiological level of CO_2 most often resulted in marked reductions in adherent growth to both silicone rubber and polystyrene (mean 84% and 86% reductions in adherence scores, respectively). However, this finding was not universal, with occasional strains adhering better when cultured in air with 5% CO_2. Enhanced adherence by strains cultured in air was shared by CNS from CAPD patients with peritonitis, and by four other clinical isolates which have been previously extensively studied (Christensen *et al.*, 1987; Schadow *et al.*, 1987). A modest fall in pH (mean 0·24 units) was noted in the cultures incubated in the CO_2 enriched atmosphere compared to those incubated in air, which may have resulted in alterations in the surface charge of both bacteria and polymers. It should be mentioned that as the pH and concentration of dissolved CO_2 in a buffered fluid are inextricably linked, caution should be exercised in ascribing changes in bacterial behaviour to only one or other of these parameters.

Adherent growth potential to silicone rubber compared to polystyrene was also found to be different. Such biomaterial specificity has been confirmed by others, and should be considered when performing adherence assays (Hogt, Dankert & Feijen, 1987). It would be wise to use materials relevant to in-vivo conditions when studying adherence *in vitro*; for example, silicone rubber with CNS from CAPD patients, because of the nature of the implanted catheter.

It was surprising that sub-MIC levels of vancomycin performed so poorly compared to cefuroxime and ciprofloxacin. Exposure to ¼ MIC of vancomycin frequently increased adherent growth to polystyrene and silicone rubber in both atmosphere conditions. Changes in the level of adherence were not only strain and antibiotic specific, but were also influenced by polymer material and atmosphere type. An earlier, smaller study did suggest that sub-MIC vancomycin exposure was not effective in reducing the adherence of CNS (Finch *et al.*, 1989).

Schadow and colleagues failed to show any inhibition in the adherence of four CNS strains which were exposed to ½ MIC of vancomycin for 6 h (Schadow *et al.*, 1987). However, the same group noted that an isolate of one of these CNS strains was more adherent after recovery from rats with experimentally-induced endocarditis treated with vancomycin, than prior to infection (Baddour, Christensen & Schadow, 1989). Evans and Holmes, in an in-vitro model of CAPD associated *S. epidermidis* biofilm, noted the failure of vancomycin to reduce bacterial viability, and the subsequent emergence of tolerant cells (Evans & Holmes, 1987). The concentration of antibiotic found deep in a biofilm layer *in vivo* may be less than the MIC for the infecting strain, therefore our results showing relatively frequent vancomycin induced increased adherence of CNS are disconcerting. Teicoplanin was found to show this effect, as might newer glycopeptides.

We attempted to correlate antibiotic and atmosphere induced changes in adherence to altered CNS surface hydrophobicity. No such relationship was demonstrable, in accordance with earlier work on CO_2 induced effects where more subtle techniques were needed to show altered cell surface physicochemistry (Denyer *et al.*, 1990). There is disagreement on the relationship between cell surface hydrophobicity and the adherence of CNS (Ludwicka *et al.*, 1984; Hogt, Dankert & Feijen, 1985; Hogt *et al.*, 1987; Schadow *et al.*, 1987). Differences in methodology may explain some of the discordant results obtained.

Changes in adherence of the CNS strains seen on exposure to antibiotics did not appear to relate to altered cell wall or membrane protein profiles. The only marked change in protein profile noted was in the cell membrane of one strain (902) exposed to cefuroxime. The nature of the two induced proteins (29·5 and 30·5 kd) is uncertain; their size is well below that of penicillin-binding proteins described to date in CNS, (Lucks & Quinn, 1985; Ubukata, Yamashita & Konno, 1985). It should be noted that exposure of Gram-negative bacteria to sub-MIC antibiotics has been shown to produce quantitative rather than qualitative changes in outer-membrane composition (Suerbam *et al.*, 1987). We have seen no evidence of cell membrane protein induction in CNS exposed to glycopeptides, in contrast with recent reports of novel proteins associated with vancomycin resistance in enterococci (Shales *et al.*, 1989; Williamson *et al.*, 1989).

Many questions concerning the pathogenesis of CNS infections remain. We feel the evidence presented to date on the influence of carbon dioxide and antibiotics on the physiology of CNS, and particularly on adherence, cannot be ignored.

Acknowledgements

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