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Influence of Carbon Dioxide on the Surface Characteristics and Adherence Potential of Coagulase-Negative Staphylococci

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Coagulase-negative staphylococci obtained from patients with continuous ambulatory peritoneal dialysis-related peritonitis were cultured in nutrient broth in an atmosphere of air containing 5% carbon dioxide (reflecting the CO_2 tension found in freshly used dialysate). Significant differences were observed between the surface chemistries of cells grown in the two atmospheres, as determined by X-ray photoelectron spectroscopy and changes in the cell wall protein profile. The growth atmosphere also influenced the adherence potential toward polystyrene and silicone in a proportion of strains examined. Thus, gaseous conditions can profoundly influence the nature of the staphylococcal surface, and this should be considered in any in vitro study of in vivo behavior.

The growth environment is known to influence significantly the properties of microorganisms, often inducing phenotypic changes characteristic of that environment (4, 18). In particular, considerable emphasis has been placed on the limited nutrients available to microorganisms in vivo at the infection site and the consequent influence this may have on growth rate, cell surface physicochemistry and composition, exopolysaccharide production, and response to antibiotics and host defenses (4, 6, 18). Interestingly, however, little attention has been given previously to the effects of gas tension in vivo on the behavior of infecting organisms, aside from the deleterious effect of oxygen on anaerobes. This may be an important oversight, since changes in cell biochemistry, surface properties, and susceptibility to phagocytosis by polymorphonuclear leukocytes have all been reported for gram-negative bacteria grown under conditions of reduced redox potential (12). More recently, oxygen-dependent exopolysaccharide production in *Pseudomonas aeruginosa* has been invoked to explain the apparently refractory nature of left-sided pseudomonal endocarditis in humans to aminoglycoside treatment (2).

Our studies have focused recently on the pathogenicity of coagulase-negative staphylococci in patients with peritonitis complicating continuous ambulatory peritoneal dialysis (19; S. P. Denyer, M. C. Davies, J. A. Evans, R. G. Finch, D. G. E. Smith, M. H. Wilcox, and P. Williams, *in* T. Wadstrom, L. Eliasson, I. Holder, and A. Ljungh, ed., *Pathogenesis of Wound and Biomaterial-Associated Infections*, in press). We are particularly concerned with identifying those features which contribute to the unique nature of this infection (3, 15). The laboratory study of staphylococcal pathogenesis in this area most frequently involves culture of organisms in air. This may be an inappropriate gaseous medium for culture if comparisons are to be made with the in vivo situation, in which higher carbon dioxide tensions are found in most body fluids and tissues (9) and variable oxygen levels are often encountered. Here we report the culture, in nutrient broth, of coagulase-negative staphylococcal strains, which were from patients undergoing continuous ambulatory peritoneal dialysis, both in air and under a carbon dioxide tension reflecting closely that found in used dialysate that was freshly obtained from patients. We subjected three strains of *Staphylococcus epidermidis* to detailed surface compositional and physicochemical analyses and compared the effects of growth in air and in 95% air with 5% carbon dioxide on these parameters. In addition, the effect of these growth conditions on the colonization potential of 50 coagulase-negative staphylococcal strains toward polystyrene and silicone surfaces was studied, since this feature may be important not only in the persistence of infection complicating continuous ambulatory peritoneal dialysis but also in the pathogenicity of coagulase-negative staphylococci.

MATERIALS AND METHODS

Collection and analysis of dialysate. Used dialysis fluid (Dianeal; Baxter Health Care, Egham, United Kingdom) from 10 different patients was collected into separate sterile containers, which were then immediately sealed. Samples were analyzed for pCO_2 within 10 min of collection by using an automated blood gas analyzer (IL1312; Allied Instrumentation Laboratory, Milan, Italy). pCO_2 levels were also measured for 10 samples of sterile nutrient broth (Oxoid Ltd., Basingstoke, United Kingdom) that were held in Bijoux bottles with loose-fitting tops. Five of these were incubated for 18 h at 37°C in air and five were incubated in a gas mixture of 5% CO_2 and 95% air.

Bacteria and culture conditions. All strains of coagulase-negative staphylococci studied were isolated from the infected dialysis fluid of patients undergoing continuous ambulatory peritoneal dialysis who were suffering from peritonitis and attending the Renal Unit, The City Hospital, Nottingham, United Kingdom. All strains were fully identified by API Staph (API, Basingstoke, United Kingdom). Stock cultures were maintained on nutrient agar (Oxoid) or blood agar (Oxoid) at 4°C. Working cultures were prepared by inoculation of colonies into nutrient broth (Oxoid) and incubation overnight at 37°C in air. Cultures for experimentation were prepared by inoculation of organisms (10^5 to 10^6 CFU) from the working culture followed by static incubation

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for the desired time at 37°C in loose-topped containers either in air or in a 5% carbon dioxide–95% air mixture.

Determination of growth. Growth curves for *S. epidermidis* 900, 901, and 904 were obtained by removing duplicate 1-ml samples from incubated cultures at various time intervals for optical density measurements at 620 nm (OD_{620}) and pour plate viable count determinations.

Elemental and functional group analysis by X-ray photoelectron spectroscopy. *S. epidermidis* 900, 901, and 904 were cultured statically for 30 h at 37°C in nutrient broth under either gas mixture and harvested separately by centrifugation (5,000 × *g* for 30 min). Individual cell pellets were washed three times and finally suspended in double-distilled water prior to freeze-drying. The freeze-dried samples were pressed firmly onto metal stubs to form a complete layer and held under vacuum at 1.33×10^{-6} Pa (10^{-8} torr) for analysis. X-ray photoelectron spectroscopy spectra were obtained by using an electron spectrophotometer (ESCALAB Mk II; VG Scientific) by using Mg Kα X rays ($h\nu$ = 1,253.6 eV). A wide-scan spectrum (1,000 eV) was recorded for each sample, together with narrow scans of the carbon 1s (C1s), nitrogen 1s (N1s), and oxygen 1s (O1s) regions. The analyzer was operated in the fixed analyzer transmission mode with pass energies of 50 eV (wide scan) and 20 eV (C1s, N1s, and O1s envelopes). Data analyses were performed on a VGS 5000 data system based on a DEC PDP 11/73 computer. The methodology used for the peak fitting of the C1s, N1s, and O1s core-level spectra has been described in detail elsewhere (14). Spectra were corrected for sample charging (referenced to C-H/C-C at 285 eV in the C1s envelope).

Physicochemical analysis. *S. epidermidis* 900, 901, and 904 were grown and harvested as described above for elemental analysis. Cell pellets were either washed three times and suspended in 1.25 mM sodium phosphate buffer to a cell density of 10^8 cells per ml for zeta-potential measurements or washed with double-distilled water and deposited onto 0.2-μm-pore-size polycarbonate membrane filters (Nuclepore Corp., Pleasanton, Calif.) to form a confluent bacterial lawn for advancing contact angle measurements (1). Zeta potentials were determined by using a Zetasizer 11 (Malvern), and the potentials were measured to a precision of 2 mV for all strains. Contact angles were determined, after a standard drying time of 60 min, by direct observation of 2-μl drops of distilled water (20°C; surface tension, 72.6 mN/m) by using a goniometer telemicroscope. Contact angles were measured with an accuracy of ±2°.

Preparation of cell wall and cytoplasmic membrane proteins. *S. epidermidis* 900, 901, and 904 were grown as described above and harvested by centrifugation (10,000 × *g* for 10 min). Bacterial pellets were washed once in phosphate-buffered saline (120 mM NaCl, 10 M Na_2HPO_4 [pH 7.4]). Cell walls and membranes were separated as described by Cheung and Fischetti (5). Briefly, bacterial cells that were collected by centrifugation of a 100-ml suspension (OD_{620}, 0.3) were suspended in 0.6 ml of digestion buffer (30% [wt/vol] raffinose, 1 mg of benzamidine per ml, and 0.5 mg of phenylmethylsulfonyl fluroride per ml in 10 mM Tris hydrochloride [pH 7.4] containing 100 μg of lysostaphin [Sigma Chemical Co., Poole, United Kingdom]). The 30% raffinose solution provides a hypertonic environment which stabilizes the protoplasts that form during lysostaphin digestion (5). The cell suspension was incubated at 37°C for 60 min, after which the protoplasts were removed by centrifugation (11,600 × *g* for 3 min). The supernatant, which contained the cell wall antigens, was stored frozen at −20°C prior to electrophoresis. The protoplast pellet was suspended in distilled water at 4°C and sonicated at 4°C for two periods of 30 s each. After lysis, membranes were collected by centrifugation (100,000 × *g* for 30 min).

SDS-polyacrylamide gel electrophoresis. Proteins were separated on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels as described previously (19). Approximately 10 μg of protein was loaded per lane. After electrophoresis, gels were fixed and stained with Coomassie brilliant blue R250 in 10% glacial acetic acid–50% methanol, subsequently destained in 40% methanol–10% glacial acetic acid, and photographed. Molecular weights were calculated from molecular size standards (molecular weight range, 12,000 to 76,000; Pharmacia LKB, Uppsala, Sweden) that were run concurrently.

Colonization of plastic surfaces. Fifty coagulase-negative staphylococcal clinical isolates were examined, and, by using a polystyrene microdilution tray system (8), bacteria (10^5 to 10^6 CFU) were grown for 18 h at 37°C in quadruplicate in 250 μl of nutrient broth in air or in a 5% CO_2–95% air mixture. For experiments with silicone rubber as the plastic, a disk (diameter, 5 mm) was aseptically placed at the base of each well and secured with sterile vacuum grease (BDH, Poole, United Kingdom) before the addition of nutrient medium. Following incubation, the contents of the wells were carefully aspirated, the wells were washed three times with normal saline, and the remaining attached bacteria were fixed with 25% Formalin and stained with crystal violet solution. Excess stain was removed by rinsing the plates in water, the plates were air dried, and OD_{565} measurements were made for each well by using an enzyme-linked immunosorbent assay plate reader (Kontron). Mean OD_{565} values were calculated and compared under the two conditions of culture relative to a line of equivalence.

RESULTS

Analysis of freshly collected, used dialysis fluid from 10 patients indicated a mean pCO_2 level of 5.02 kPa (range, 4.20 to 5.42 kPa; standard error of the mean, 0.12 kPa); incubation of nutrient broth in an atmosphere of 5% CO_2–95% air achieved similar mean pCO_2 levels (5.03 kPa; range, 4.56 to 5.50 kPa; standard error of the mean, 0.10 kPa). By this mechanism, it was possible to achieve a CO_2 gas tension in nutrient broth closely approaching that which is likely to exist in the dialyzed peritoneum. By using this approach, the influence of increased CO_2 gas tension could be explored with reference to sterile nutrient broth incubated in air (mean pCO_2; 0.65 kPa; range, 0.61 to 0.69 kPa; standard error of the mean, 0.01 kPa).

Figure 1 shows the growth curves of the three *S. epidermidis* strains, 900, 901, and 904, in nutrient broth under both atmospheric conditions; the pattern of growth was similar for both optical density and viable count measurements. While a higher growth yield was achieved for all strains in 100% air, the shape of the growth curve, while strain specific, did not differ significantly for organisms grown under the different gas compositions. Thus, strains harvested at similar times from either condition of growth are at similar stages of their growth cycle and can be readily compared.

An examination of the physicochemical surface characteristics of the three staphylococcal strains (Table 1) distinguished between strains 900 and 901 on the basis of contact angle but not surface charge; strain 904 was of intermediate character. The surface of strain 901 can thus be considered the most hydrophobic (17), in agreement with our earlier

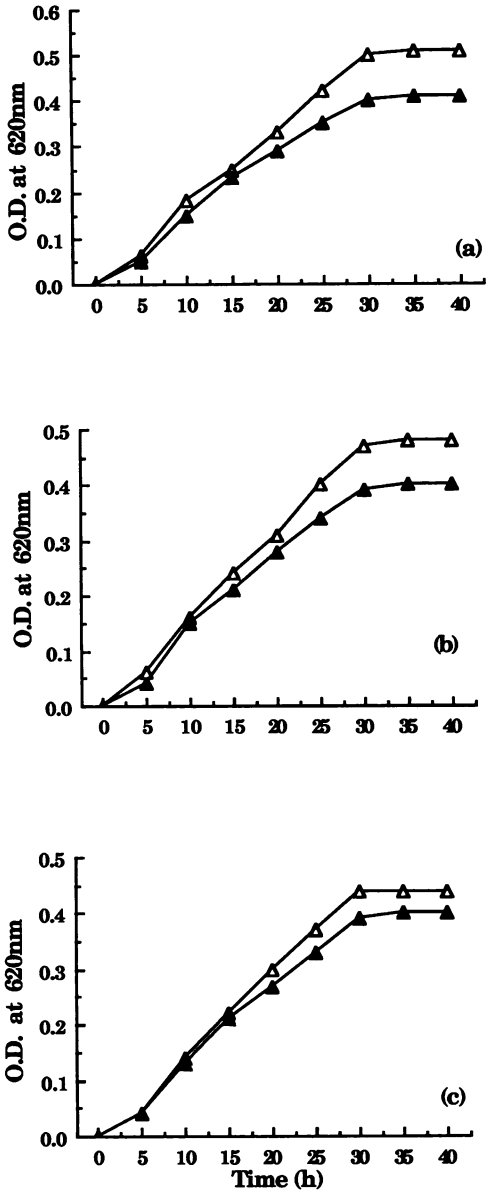

FIG. 1. Comparison of growth curves of *S. epidermidis* 900 (a), 901 (b), and 904 (c) cultured in 100% air (△) and 5% CO_2–95% air (▲).

findings of enhanced adhesion of this strain to hydrophobic polystyrene (S. P. Denyer, M. A. Khan, and M. C. Davies, Abstr. 5th Int. Symp. Rapid Meth. Automation Microbiol. Immunol., p108, 1987). The strain-specific surface physicochemical characteristics were maintained irrespective of the growth conditions.

Only minor differences in elemental composition existed between the *S. epidermidis* strains. However, a clear decrease in surface carbon content with an accompanying increase in oxygen and nitrogen levels led to a significant increase in O/C and N/C atomic concentration ratios for all strains when they were grown in the CO_2-air mixture (Table 2). It is likely that this represents an increase in surface exposure of both carbohydrate and protein (16) at the expense of some other hydrocarbon-containing component(s). Indeed, functional group analysis would suggest that

TABLE 1. Physicochemical characteristics of three *S. epidermidis* strains grown in nutrient broth under different gaseous conditions

Strain	5% CO_2–95% air		100% air	
	Zeta potential (mV)	Contact angle (degrees)	Zeta potential (mV)	Contact angle (degrees)
900	−37	15	−34	15
901	−40	26	−40	27
904	−36	24	−36	24

growth under 5% CO_2–95% air results in a marked decrease in C—C bonds with a concomitant rise in C—O and C=O bonds (data not shown).

The cell wall and membrane protein profiles of *S. epidermidis* 900, 901, and 904 grown either in air or in the CO_2-air mixture were examined by SDS-polyacrylamide gel electrophoresis (Fig. 2). While no changes in the cell wall proteins of strain 901 were noted, several changes occurred in strains 900 and 904. In particular, a protein of 67 kilodaltons that was present in air-grown cells was repressed in the CO_2-air mixture (Fig. 2a; compare lanes A and E with lanes B and F, respectively), while the converse was observed for a 53-kilodalton protein. In addition, for strain 900, proteins of 76 and 46 kilodaltons were induced in the presence of CO_2 (Fig. 2a; compare lanes A and B). Neither the functionality nor the location of any of these proteins has yet been determined. Little difference was observed in the cell membrane profiles of all three strains grown under either culture condition (Fig. 2b).

Figures 3a and b compare the effects of the growth atmosphere on the colonization of silicone and polystyrene surfaces by 50 strains of coagulase-negative staphylococci during growth in nutrient broth. In general, within the collection of strains used, colonization of silicone was more successful than was colonization of polystyrene, and the mean level of adherent growth with silicone was independent of the gaseous conditions, as indicated by the uniform distribution of strains about the line of equivalence (Fig. 3a). In the case of polystyrene, however, growth in 100% air generally produced better colonization than did growth in the CO_2-air mixture (Fig. 3b). On an individual strain basis, the gaseous condition can significantly ($P < 0.05$ by Student's t test) influence the level of adherent growth of selected strains. These are highlighted in Fig. 3, and attention is drawn to the behavior of strains 900, 901, and 904 that were examined here in detail.

DISCUSSION

A superficial comparison based on staphylococcal growth and gross physicochemistry would suggest that CO_2 has no major effect on the staphylococcal cell. A more detailed

TABLE 2. Atomic concentration ratios for three *S. epidermidis* strains grown in nutrient broth under different gaseous conditions

Strain	5% CO_2–95% air		100% air	
	N/C	O/C	N/C	O/C
900	0.175	0.434	0.160	0.355
901	0.207	0.433	0.162	0.356
904	0.188	0.416	0.160	0.364

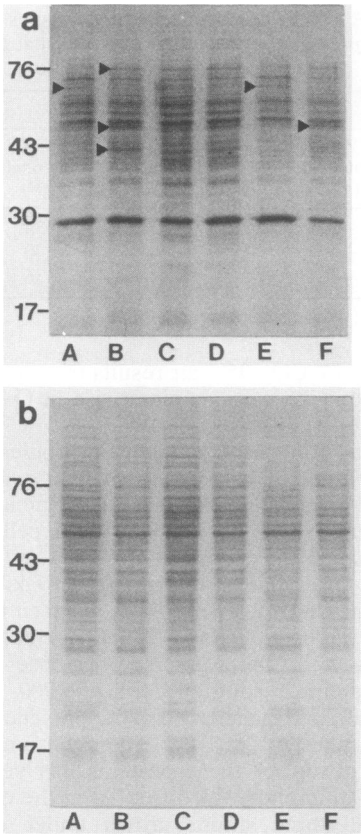

FIG. 2. (a) SDS-polyacrylamide gel electrophoresis of the cell wall proteins of *S. epidermidis* 900 (lanes A and B), 901, (lanes C and D), and 904 (lanes E and F) grown in nutrient broth in air (lanes A, C, and E) or in a CO_2-air mixture (lanes B, D, and F). Molecular masses are given in kilodaltons. (b) SDS-polyacrylamide gel electrophoresis of the cytoplasmic membrane proteins of *S. epidermidis* 900 (lanes A and B), 901 (lanes C and D), and 904 (lanes E and F) grown in nutrient broth in air (lanes A, C, and E) or in a CO_2-air mixture (lanes B, D, and F). Molecular masses are given in kilodaltons.

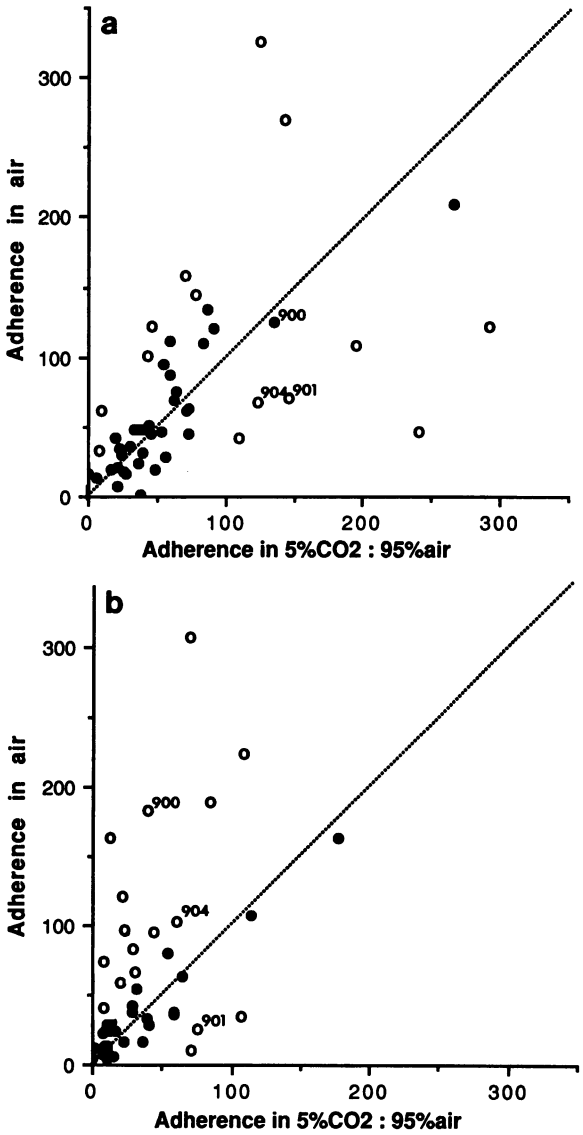

FIG. 3. Relationship of adherent growth to silicone (a) and polystyrene (b) of 50 coagulase-negative staphylococcal strains in 100% air and 5% CO_2–95% air. Units of adherence are mean optical densities multiplied by 1,000 (see text). Dotted lines represent lines of equivalent surface colonization. Open circles represent strains whose adherent growth was markedly affected by gaseous conditions, causing a significant ($P < 0.05$) deviation from the line of equivalence.

analysis of the surface chemistry and protein composition, however, indicates fundamental differences between cells grown in nutrient broth in 100% air and those grown under a 5% CO_2–95% air mixture. X-ray photoelectron spectroscopy suggested that there is a significant increase in surface-exposed carbohydrates and proteins, and confirmation of changes in cell wall protein profiles was given by gel electrophoresis.

Adherence behavior is polymer dependent, as reported by other investigators (10) and, in the case of polystyrene, is atmosphere dependent. With silicone, CO_2 tension did not affect the mean adherence of the *S. epidermidis* isolates, but some individual strains exhibited atmosphere-related differences (strains 901 and 904, for instance). It is likely that successful adherence requires an appropriate balance between surface physicochemical and chemical characteristics; the influence of CO_2 on this balance may be complex and could cause the different adherences seen between the two polymers in the two atmospheres.

The basis of the observed staphylococcal surface changes is open to speculation. Conceivably, differences in growth atmosphere, in particular with respect to carbon dioxide tension, could affect the balanced functioning of metabolic pathways and the induction and repression of enzyme systems (7), thereby altering the biosynthesis and arrangement of surface components. CO_2 has been shown to induce the synthesis of α-toxin in *Staphylococcus aureus* (11). A lowered redox potential and a consequent greater degree of anaerobiosis has been advanced as a possible explanation of increased formation of a serum high-density lipoprotein-binding component in the cell wall of *Staphylococcus capitis* (13). In addition, a small drop in pH (0.5 units to pH 6.6) was noticed in nutrient broth in the presence of CO_2, which may also possibly influence metabolic and surface characteristics. Whatever the cause of these changes, the inescapable conclusion is that in vitro models must consider the in vivo gaseous tension at the infection site. Duplication of these conditions may be essential in order to reveal the true

physiological characteristics, including the surface composition, of an organism. The significance of this fact in the study and understanding of surface-related behavior is demonstrated in the adhesion and colonization studies reported here. The effect of CO_2 tension on the pH and composition of peritoneal dialysis fluid and their influence on staphylococci grown in vitro in this biological fluid will be considered in a further report (M. H. Wilcox, D. G. E. Smith, J. A. Evans, S. P. Denyer, R. G. Finch, and P. Williams, submitted for publication).

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