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AN ABSTRACT OF THE THESIS OF Abbas Sadeghi for the Master of Science in Biology presented August 3, 1992.

Title: Development of a Semi-synthetic Medium Supporting Adherent Growth in Coagulase-negative staphylococci.

APPROVED BY THE MEMBERS OF THE THESIS COMMITTEE:

Mary L./Taylor, Chair

Richard Peterson

Gordon Kilgour

A semi-synthetic medium for use in determining adherent growth with Staphylococcus epidermidis and Staphylococcus saprophyticus was developed. Production of an adherent biofilm was dependent upon the presence of hematin in the growth medium. Clinical strains of Staphylococcus epidermidis were tested for production of an adherent biofilm in trypticase soy broth, the semi-synthetic medium and the hyperalimentary nutrient solution used in the neonatal hospital unit. An adherent biofilm was obtained when

Staphylococcus epidermidis was cultured in hematin supplemented hyperalimentary solution. Growth in the hyperalimentary nutrient solution diluted with fetal calf serum showed the same growth rate as when the nutrient solution was diluted with water. The final growth yield was always higher in serum diluted nutrients. There was no effect of hematin on the growth rate of the organisms.

DEVELOPMENT OF A SEMI-SYNTHETIC MEDIUM SUPPORTING
ADHERENT GROWTH IN COAGULASE-NEGATIVE STAPHYLOCOCCI

by

ABBAS SADEGHI

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University
1992

TO THE OFFICE OF GRADUATE STUDIES:

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INTRODUCTION

After many years of investigations, coagulase-negative staphylococci which were formerly considered commensal organisms, are known to be opportunistic pathogens of foreign bodies (Christensen et al, 1982). These investigators reported that the introduction of foreign bodies in humans, particularly prosthetic cardiac valves, cerebrospinal fluid shunts, orthopedic appliances, and intravascular catheters, predisposed the patient to infection. It has been postulated that the adherence of the bacteria to the surface of these medical devices, is the critical determinant for infection. Bayston and Penny (1972) reported that the growth of coagulase-negative staphylococci produced a mucoid deposit on the cerebrospinal fluid shunts. This deposit could be visualized by staining with alcian blue which is known to react with acidic polysaccharides (McKinney, 1953). In recent years, scanning electron microscopic studies of naturally infected medical appliances have demonstrated the adherent growth of Staphylococcus epidermidis (Russell et al, 1987). Using the normal mouse model, Christensen et al (1983) showed that intracutaneous injection of coagulase-negative staphylococci resulted in no infection. However, introduction of the coagulase-negative staphylococci to mice that had intravascular catheters implanted subcutaneously resulted in an infection at the catheter site in one-third of the mice.

In recent years numerous investigators have noted an increasing incidence of coagulase-negative staphylococcal infections of medical devices. Christensen et al (1982) found that many of these coagulase-negative staphylococcal strains are able to coat the walls of culture tubes with a bacterial film (biofilm) or slime. Intravascular catheters immersed in culture broth containing coagulase-negative staphylococci also showed a biofilm (Russell et al, 1987). A comparison of the staphylococcal isolates from patients with catheter-associated infection showed that these isolates produce more slime than do laboratory strains (Christensen et al, 1982). They speculated that mucoid growth is important in the pathogenesis of catheter-associated infections. Christensen (1985) has proposed that these microorganisms infect medical appliances because of a natural inclination to adhere to the surface of the appliance (Christensen et al, 1985).

Electron micrograph studies of the adherence of S. epidermidis to plastic catheters indicate that initially small numbers of organisms lodge in irregularities along the catheter surface from which micro- and macro- colonies build up. The colonies are assumed to adhere to the surface by slime which does not have the fibrillar characteristic of fibrin (Christensen et al, 1982). Examination of electron micrographs from the biofilm coat on catheter surfaces indicates that multiple layers of staphylococcal cells are embedded in the biofilm. Isolates were made from these biofilms for in vitro studies. They found that the strains were able to adhere to the smooth surfaces

and produce biofilms. Thus, Peters et al (1984) proposed that the biofilm production could be important as a staphylococcal defense mechanism against the host immune system.

In analogous in vitro experiments, Streptococcus mutans is found to adhere to dental surfaces by means of a tightly bound cell surface protein in the initial attachment. The production of glucan from sucrose by extracellular glucosyl transferases facilitates the adherence of these bacteria and plays a major role in the bacterial aggregation which makes up plaque. It is believed that Streptococcus mutans adherence occurs in two phases by two different mechanisms. First, there is rapid attachment to the tooth enamel followed by slow cellular accumulation and microcolony formation mediated by cell surface carbohydrates (Staat et al, 1980). It is possible that the adherence of Streptococcus mutans to dental surfaces may resemble the adherence of coagulase-negative staphylococci to smooth surfaces of implanted foreign bodies. Although the S. epidermidis slime is not a glucan, it might function in a similar manner as either an adhesin (providing permanent binding to foreign bodies) or as a cohesin, binding cells together on the foreign body surfaces (Christensen et al, 1982).

Many bacteria, for example, Streptococcus mutans (McBride et al, 1984), Neisseria gonorrhoeae (Swanson, 1978), and Escherichia coli (Isaacson, 1985), express their adhesin differentially. Therefore, at all times the bacterial population consists of a mixture of phenotypes with different adhesive properties. Considering this observation, it is

possible that slime-producing strains of S. epidermidis also exhibit a mixture of adhesive phenotypes. Phenotypic variation in the display of virulence and colonization factors has been described in many adhesive systems. N. gonorrhoeae (Swanson, 1972), as an example of gram-negative coccus, produces two adhesive systems: pili and type II outer membrane protein. Either or both of these systems may or may not be present in the organism. The ability to colonize and cause disease is influenced by the presence of one or both of these systems. E. coli is able to display many forms of fimbriae. E. coli producing fimbriae which are Type 1 or 987 p exhibit variation between fimbriated forms and non-fimbriated forms depending on the growth condition. S. mutans is able to bind the dental surfaces by glucan production in a sucrose-rich medium. The adherence of S. mutans is greatly dependent upon the hydrophobicity of the outer cell surface. Some investigators believe that there is no variation in glucan production but that variants with diminished surface hydrophobicity and adherence ability occur in S. mutans (Christensen et al., 1987).

In 1988, Christensen et al. reported that pathogenic strains of S. epidermidis exhibit a mixed population of colony phenotypes when grown on a high-salt, low-glucose agar (Memphis agar). The isolation of a small colony variant from this medium has directed the attention to the role of these colony morphotypes in the pathogenesis of foreign body-associated infections. S. mutans also adheres to glass when grown in sucrose-rich medium, and exhibits a distinct colonial morphology when grown on mitis salivarius agar (Murchison et

al,1981). Virulence studies with the coagulase-negative staphylococcal variant phenotypes indicate that the small variant forms also exhibit less virulence in the development of rat endocarditis.

Recent interest in S. epidermidis adherent growth was stimulated by the reports of Christensen et al (1982) that strains isolated from patients with catheter-related infections formed mucoid material on solid surfaces in static cultures or on intravascular catheters. Testing for adherent growth is claimed to be a useful marker for clinically significant infections with coagulase-negative staphylococci. These findings suggest that adherent growth is a stable trait for certain strains of coagulase-negative staphylococci. Moreover, slime production (adherent growth positive) is a relative phenomenon, with some strains producing more slime than others in response to specific environmental conditions (Davenport et al, 1986).

Many factors including catheter composition and bacterial adherence characteristics may affect catheter colonization and incidence of catheter-related infections (Russell et al, 1987). Many studies also have shown that factors such as the duration of insertion, the experience and technique of the personnel introducing the catheter, the length of the catheter inserted, the occurrence of clots, and the use of topical or systemic antibiotics in the colonization and catheter-associated infections. Peters et al (1982) observed preferential attachment of S. epidermidis to catheter surface irregularities by means of polysaccharide material surrounding

individual cells. However, recent studies have shown an enhanced adherence of S. epidermidis to heparin-bounded catheters treated with fibronectin. It is speculated that heparin may provide a substrate for serum factors such as fibronectin which can modulate bacterial adherence (Russel et al, 1987).

The long-term use of intravenous catheters which is an accepted method of providing nutrition for low-birth weight infants, has been associated with serious complications (thrombosis, perforation, infection, etc.). Hickman and Broviac catheters are also used in long-term venous access for the treatment of patients that are seriously ill. Heparin is usually used to decrease the incidence of thrombosis (Russel et al, 1987). In 1988, McInntyre et al. reported that disodium ethylenediaminetetraacetic acid (EDTA), a chelating agent which acts to prevent coagulation of blood, inhibits the growth of S. epidermidis. EDTA is also considered to attack cell membrane and disrupt cellular integrity in gram-negative bacteria but its effect on gram positive bacteria is unknown. In McInntyre's report, comparing EDTA efficiency with those of heparin alone and a vancomycin-heparin preparation in vitro, they concluded that EDTA could prove to be a safe and effective means of catheter maintenance.

Recent progress in the understanding of bacterial adhesion mechanisms at the molecular level has provided some information concerning antibiotic modulation of adhesion and surface physico-chemical properties determining bacterial adherence ability. Bruce et al. reported an increase in surface hydrophobicity due to slime

production in a pathogenic strain of S. epidermidis (Bruce et al, 1987). In 1988, Christensen et al. observed decreased bacterial adhesion and also reduced surface hydrophobicity in β -lactam treated coagulase-negative staphylococci.

Exposure of Staphylococcus aureus and Streptococcus pyogenes to β -lactam antibiotics results in the release of lipoteichoic acid (LTA) into the medium (Schifferli and Beachey, 1988). The decrease in the surface hydrophobicity of penicillin-treated streptococci is speculated to be due to LTA release. Moreover, the LTA released by penicillin-treated S. pyogenes is paralleled by a reduction in bacterial adhesion to epithelial cells. These results suggest a major role of LTA in streptococcal adhesion to host proteins such as fibronectin. Another protein or carbohydrate is considered to bind fibronectin in S. aureus (Schifferli and Beachey, 1988). In addition, clindamycin treatment decreases the binding of fibronectin to S. aureus but not to S. pyogenes, suggesting the inhibition of synthesis of fibronectin receptors on S. aureus (Schifferli and Beachey, 1988).

McKinney (1953) reported specific staining of bacterial polysaccharide by means of a cationic dye (alcian blue). They found that at neutral or basic conditions only acidic carbohydrates showed retention of the dye. Jones et al (1963) observed a high incidence of slime production only when S. epidermidis strains were cultured with low concentration of glucose (less than 0.3%) or pyruvate as the energy source. No slime was detected when the strains were cultured with other energy sources.

Adherent growth has been proposed to be a major virulence factor. Slime production always has been reported for cultures grown in trypticase soy broth or other laboratory media where conditions were not physiological. This work was conducted to make a semi-synthetic medium which would support adherent growth in coagulase-negative staphylococci. Attempts were made to simulate in vitro bacterial growth under in vivo conditions.

Strains of coagulase-negative staphylococci were provided by Dr. W.A. Simpson, University of Tennessee College of Medicine, Memphis, Tennessee. Thirty-seven clinical isolates of coagulase-negative staphylococci were received from clinical sources of patients hospitalized in various hospitals in Memphis, Tennessee. All isolates were gram-positive, catalase-positive, coagulase-negative staphylococci. *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus saprophyticus* (ATCC 29522), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus carnosus* (ATCC 25919), *Staphylococcus epidermidis* (ATCC 12228), and *Staphylococcus saprophyticus* (ATCC 29522) were used for comparative studies. Working stock cultures were maintained on trypticase soy agar. Stock cultures were maintained in glycerol stocks which were stored at -20°C.

SECTION 1. MATERIALS

Cultures of slime-producing and non-slime-producing strains of coagulase-negative staphylococci were grown in trypticase soy broth (Difco); or the following media prepared from nutrient agar (Difco):

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE

A slime-producing strain of Staphylococcus epidermidis (RP62A) and a non-slime-producing strain (Ham 892) derived from RP62A were provided by Dr. W.A. Simpson, University of Tennessee College of Medicine, Memphis, Tennessee. Thirty-seven clinical isolates of coagulase-negative staphylococci were collected from blood cultures of incubated neonates at Emanuel Hospital, Portland, Oregon. All isolates were gram-positive, catalase-positive coagulase-negative staphylococci. Staphylococcus sciuri (ATCC 27840), Staphylococcus cohnii (ATCC 29972), Staphylococcus xylosus (ATCC 29966), Staphylococcus epidermidis (ATCC 12228), and Staphylococcus saprophyticus (ATCC 15305), were used for comparative studies.

Working stock cultures were maintained on trypticase soy agar. Stock cultures were maintained as glycerol stocks which were stored at -20°

C

BACTERIAL GROWTH

Cultures of slime-producing and non-slime producing strains of coagulase-negative staphylococci were grown in trypticase soy broth (Difco); or the following media prepared from neonatal intravenous

solutions formulated by the Emanuel Hospital Pharmacy. The composition of this solution is shown in Table I.

TABLE I
COMPOSITION OF NEONATAL NUTRIENT

Nutrient	Volume (ml)
Dextrose (70%)	55.71
Trophamine* (10%)	75.00
NaCl (2.5 mg/ml)	2.40
K-acetate (2 mg/ml)	3.00
K-phosphate (1 mM/ml)	3.00
Calcium (9 mg/ml)	12.00
MgSO ₄ (0.4 mg/ml)	3.00
Pediatric multiple vitamins**	3.75
Neotrace	3.75
Heparin (100 Units/ml)	3.00
Sterile water	135.39
Total	300.00

* Kendall McGow Laboratories Inc. balanced amino acids solution.

** A mixture of different vitamins for neonates.

The neonatal intravenous solution was mixed to give the following media:

- Neonatal nutrient-water (NNH). This medium contained 50 ml neonatal nutrient plus 50 ml sterile distilled water.
- Neonatal nutrient-serum (NNS). This medium contained 50 ml neonatal nutrient plus 50 ml fetal calf serum.
- Neonatal nutrient-water-lipid (NNHL). This medium contained 50 ml neonatal nutrient plus 43 ml sterile distilled water and 7 ml of sterile pharmacy formulated lipid.

d) Neonatal nutrient-serum-lipid (NNSL). This medium contained 50 ml neonatal solution plus 43 ml fetal calf serum and 7 ml of sterile pharmacy formulated lipid.

TABLE II
COMPOSITION OF U MEDIUM

Components	amount/liter
Vitamin free casamino acids	3.00 g
K ₂ HPO ₄	8.90 g
KH ₂ PO ₄	1.70 g
NaCl	2.00 g
Tryptophane (b)	0.04 g
Cysteine (b)	0.024 g
Thiamine (a)	0.002 g
Vitamin stock solution A (c)	10.00 ml
Vitamin stock solution B (d)	2.00 ml
Glucose (a)(50% sterile solution)	5.00 ml
Salts stock (e)	20.00 ml
Total	1000.00 ml

- a Glucose, salts, and thiamine were each autoclaved separately and added to the sterile base medium.
- b Cysteine and tryptophane were filter-sterilized and added to the sterile base medium
- c Vitamin stock A: pyridoxal HCl 0.025 g; thiamine 0.100 g; calcium pantothenate 0.100 g; riboflavin 0.100 g; niacin 0.100 g; p-amino benzoate 0.050 g; pyridoxine HCl 0.200 g; vitamin B₁₂ 200 ug; water 1000ml.
- d Vitamin stock B: biotin 500 ug; folic acid 25 ug; water 200 ml.
- e Salt stock: sodium citrate 50 g; MgSO₄ .7H₂O 10 g; CaCl₂.2H₂O 1 g; water 1000 ml.

A semi-synthetic medium ("U") was formulated as described in Table II. Various concentrations of filter-sterilized hematin were added to U medium as specified in each experiment. The hematin was

prepared as described by Clarke and Knowles (1980). Agar media were prepared by addition of 15 g agar/liter.

For oxygenation of cultures, 10 ml of the appropriate medium was placed in 125 ml Erlenmeyer flasks. After inoculation the flasks were incubated at 37° C on a gyrotory shaker at 100 rpm. Growth was monitored by following the increased turbidity on a Klett-Summerson photoelectric colorimeter using a number 66 filter or by viable cell count. Anaerobic conditions were generated using the GasPak systems (BBL Microbiology systems) with H₂-CO₂ atmosphere. Cultures were stocked on TSB agar.

MICROBIAL ADHERENCE

The qualitative assessment of adherent growth was determined as previously described (Christensen et. al, 1982). This tube method consisted of inoculating 7 ml of trypticase soy broth (TSB), the semi-synthetic medium (U broth), NNH or NNS with a loopful of bacteria from a trypticase-soy agar plate culture. The broth culture tubes were incubated for 24 h at 37° C. The contents of the tubes were poured off and the adherent growth on the test tube wall was fixed, and stained with alcian blue.

CHEMICALS

All chemicals were of the highest purity or analytical grade.

Trypticase Soy Broth, Vitamin-free casamino acids, and Bacto-agar were obtained from Difco (Microbiology systems, Difco Inc.). Cysteine-HCl, tryptophane, thiamine, biotin, folic acid, and hematin were obtained from Sigma Chemical Company, Saint Louis, MO. Alcian blue (Chrome-Gessellschaeft) was obtained from Polyscience Inc., Warrington, PA. Fetal calf serum was obtained from Gibco Corporation.

RESULTS

ADHERENT GROWTH BY COAGULASE-NEGATIVE STAPHYLOCOCCI

A total of 37 strains of coagulase-negative staphylococci were obtained and tested for the ability to produce a biofilm of adherent growth on test tube walls. The organisms were cultured in either TSB, or hematin supplemented U broth and the test tubes were stained after growth of the culture as outlined in Materials and Methods. The test for slime production was easy to perform and interpret. The coating of standard glass test tube walls by an adherent film of S. epidermidis RP62A and three clinical isolates is shown in Figure 1a. 37 clinically isolated strains were checked for the ability to produce slime. As shown in Table IV, strains that produced an adherent film ranged from a weak to very strong positive. Adherent growth was compared to S. epidermidis RP62A which gave the most positive adherent growth. The non-adherent control was S. epidermidis Ham 892. Each strain was tested four times and examined by three different observers. Although there were a few strains that showed unstable, weakly adherent biofilm, the strongly biofilm-adherent strains remained stable over a period of four to six months. It can be seen that 11 out of 37 clinical strains (30%) produced adherent biofilms. This tenacious slime was difficult to remove even by scrubbing with a test tube brush.

Figure 1. Adherence of *S. epidermidis* grown in broth to the walls of culture tubes. The tubes were emptied and stained with alcian blue. (a, c) non-adherent strain: (b, d) weak adherent strain in TSB: (f) strong adherent strain in TSB.

When a slide prepared from adherent or non-adherent cells was stained with alcian blue, only the cells stained blue.

TABLE III

QUALITATIVE ASSESSMENT OF BACTERIAL ADHERENCE TO CULTURE TUBE WALLS

strain	Adherence	
	TSB	hematin supplemented U-broth
<u>S. sciuri</u>	-	-
<u>S. cohnii</u>	-	-
<u>S. epidermidis</u>	+	+
<u>S. xylosum</u>	-	-
<u>S. saprophyticus</u>	+	+
<u>S. epidermidis</u> -1000	+	+
4350-1	+	+
4350-2	++	++
9284	++	++
8894	++	+
9976	-	-
0282509	+	+
8542	-	-
9134	-	-
1634-1	++	+
7613	-	-
6257	+	+
2236	+	+
2161	++	++
2289	+	+
6557-2	+	+
<u>S. epidermidis</u> RP62A	++	++
<u>S. epidermidis</u> Ham 892	-	-

Symbols: ++, strong adherence: +, weak adherence: -, no adherence.

The absence of extracellular blue staining material in the adherent cell suspension indicates that the adherent growth is associated with the cell. Adherent growth from clinical isolates was only obtained

with the coagulase-negative isolates that systematically keyed to S. epidermidis or S. saprophyticus.

Diebel and Jones (1962) worked with carbon sources that would support anaerobic growth of S. epidermidis. Using a semi-synthetic medium, they observed that several carbon sources would support anaerobic growth but only glucose gave slimy cultures. We also have found that adherent growth was obtained only when glucose was the carbon and energy source. The carbon and energy sources substituted for glucose were 0.3% glycerol, 0.1% serine, 0.5% sodium pyruvate or uracil.

Christensen (1987) has reported that adherent growth of S. epidermidis was obtained only when low concentration of glucose was used. However, the neonatal nutrients present in the catheters contain 13% glucose and this probably results in a high concentration of glucose at the blood vessel-catheter junction. If adherent growth is a virulence factor then it would be expected that all coagulase-negative clinical isolates would produce adherent growth.

Our goal was to devise a semi-synthetic medium that would be more physiologically oriented than TSB. The "U" medium we devised supported good growth of all clinical isolates, but no adherent growth could be demonstrated with the known adherent strain, RP62A or any of our clinical isolates. However, as shown in Table III and IV, growth of some clinical isolates and the known strains of S. epidermidis and S. saprophyticus in hematin supplemented U broth gave adherent growth comparable to that obtained when the strains

were grown in TSB. It can be seen that the presence of hematin at 0.5 mg/100ml gave results equivalent to those obtained in TSB.

TABLE IV

A COMPARISON OF THE QUALITATIVE ASSESSMENT OF ADHERENT GROWTH IN TSB TO THAT IN U MEDIUM SUPPLEMENTED WITH HEMATIN

Strain	Adherence				
	U broth + hematin (mg/100ml)				
	TSB	0.00	0.02	0.5	1
4350-1	+	-	±	+	+
4350-2	++	-	±	++	+
9284	++	-	±	++	+
8894	++	-	±	++	+
6257	++	-	±	++	++
2289	+	-	+	±	±
2236	+	-	-	±	±
9134	-	-	-	-	-
<u>S. epidermidis</u> (RP62A)	++	-	+	++	+
<u>S. epidermidis</u> (Ham 892)	-	-	-	-	-
<u>S. saprophyticus</u>	+	-	-	+	+

Symbols: ++, strongly adherence; +, weakly adherence; -, no adherence; ±, adherent growth barely visible.

It appears that for some strains adherent growth is decreased when hematin concentration is raised to 1 mg/100ml. Thus it would appear that physiological concentrations of hematin or some other blood factors may facilitate adherent growth.

In order to determine if adherent growth could occur with nutrients administered under physiological conditions, hyperalimentary nutrients were diluted 1:1 with sterile distilled water

(NNH) or fetal calf serum (NNS). The production of adherent growth was tested using static cultures as described in Materials and Methods. Neither the adherent-positive control S. epidermidis, RP62A, nor any of the clinical isolates produced adherent growth in these media. Since lipids also are administered into the intravenous tube when feeding neonates, adherent growth was checked using NNH plus lipid and NNS plus lipid as described in Materials and Methods. Again no adherent growth was obtained. However, the addition of 5.0 ug/ml hematin to the media resulted in adherent growth in all isolates that showed adherent biofilm in TSB. The heme concentration present in neonates is not known but the range for adults is approximately 0.1 mg/100ml. Thus heme could be a physiological component that contributes to bind Staphylococcus epidermidis to surfaces resulting adherent growth. When NNS and NNH cultures, with and without lipid that were incubated with shaking, they did not produce adherence to the walls of the flasks under any growth condition tested. However, there was a heavy ring of growth at the air-liquid interface of all cultures that contained neonatal nutrients. Qualitatively this growth was much heavier than that found with stationary cultures. Aerated cultures grown in "U" medium did not give the adherent ring at the air-liquid interface, but the cultures became very flocculated. As would be expected oxygenation results in much heavier growth for all cultures. A similar finding was obtained when the strains were tested for phenotypic variation as described by Christensen et al. (1987). Using a low glucose-high salt MA medium (Memphis agar) they

demonstrated that adherent and non-adherent strains of S. epidermidis showed different colony morphotypes. Clinical isolates that showed adherence to the walls of the test tubes were compared for the ability to exhibit phenotypic variation cultured on Memphis agar, U agar, and hematin supplemented U agar. As shown in Table V, all strains tested showed no correlation between adherence to test tube walls and the ability to produce different morphotypes on Memphis agar and hematin supplemented U medium. Thus while the addition of hematin to U broth results in adherent growth, it had no effect on the formation of different colony morphotypes.

ADHERENCE TO PLASTIC TEST TUBES OF VARIOUS CHEMICAL COMPOSITION

Several investigators have reported that a positive test for adherent growth depends upon the composition of the solid surface (Russell et al., 1987; Christensen et al., 1985). Thus S. epidermidis RP62A, the slime-negative control S. epidermidis, Ham 892, and an adherent positive clinical isolate, 8894, were cultured and tested for ability to adhere to a variety of surfaces.

Falcon snap-cap polystyrene test tubes, Fisher brand snap-cap polystyrene test tubes, and polybutadiene conical test tubes were incubated in 500 ml Erlenmeyer flasks containing 150 ml of TSB inoculated with RP62A, Ham 892, or the clinical isolate, 8894. The

TABLE V
PHENOTYPIC VARIATION ON MA AND HEMATIN SUPPLEMENTED U AGAR

Strain	Adherence	Medium	
		MA	U
4350-1	++	uf	uf
4350-2	+	vf	vf
9284	++	uf	vf
8894	++	uf	vf
6257	+	uf	vf
6557-2	+	vf	uf
9976	+	uf	uf
2161	++	uf	vf
0282509	+	vf	uf
<u>S. epidermidis</u> -1000	+	uf	uf
<u>S. epidermidis</u> RP62A	++	vf	vf
<u>S. epidermidis</u> Ham89	-	vf	vf

Symbols: ++, strong adherence: +, weak adherence: uf, no phenotypic variation: vf, variant forms.

flasks were shaken at 100 rpm at 37° C for 24-48 hrs. The suspended tubes were removed and stained as described in Materials and Methods. Although adherent growth producing strains (8894 and RP62A) barely showed visible adherence growth at the artificial irregularities of the tube walls, the overall assessment of adherence on plastic smooth surfaces was negative. When a duplicate set of flasks with test tubes was inoculated and incubated in static cultures, adherent growth was supported in both the clinical isolate 8894 and S. epidermidis RP62A. Qualitative assessment of adherent material appeared to be the same regardless of the wall composition. The negative control, Ham 892, did not produce adherent growth in test tubes of any material. While there was no adherent biofilm on the

always developed a heavy wall-attached culture at the liquid-air interface.

GROWTH CHARACTERISTICS

The growth of 11 coagulase-negative staphylococci were monitored as described in Materials and Methods. Most isolates tested showed higher density when cultured in the presence of serum (NNS) than when cultured in either TSB or NNH. Two clinical isolates, 8894 (adherent positive) and 9134 (adherent negative), were studied to determine if one had growth rate advantage. S. epidermidis RP62A was used as a comparative control. As shown in Figure 2, the adherent positive strain showed parallel growth rates when cultured in NNS, NNH, TSB. As shown in Figure 4, the non-adherent strain, 9134, grew at the same rate and to the same density when cultured in TSB or NNS. However, this strain did not grow well in neonatal nutrient in the absence of serum. As shown in Figure 3, the control strain, RP62A, showed parallel growth rate in NNS, NNH, and TSB but the final growth yield in TSB was greatly reduced. The growth of all strains tested indicated that the presence of lipid with the neonatal nutrient inhibited the final growth yield (Figure 5, 6, 7). The ability or inability to produce adherent growth did not appear to be related to the final culture density.

Many of our clinical isolates were coagulase-negative staphylococci that would not key to Staphylococcus epidermidis.

Therefore, we tested the comparative growth of several non-S. epidermidis coagulase-negative staphylococci in TSB. As shown in Figure 8, each strain had a different generation time varying from 90 to 114 minutes. This compares to a generation time of approximately 90 minutes for the non-adherent 9134 and adherent RP62A and 8894. Two ATCC strains, S. hemolyticus and S. hominis were chosen to study the growth in the more physiological medium, NNS. As shown in Figure 9, the rate was the same in TSB and NNS. Since U medium did not support slime production but U medium plus hematin did (Table I), we compared the growth of the known slime producer RP62A on these two media. As shown in Figure 10 there was no difference in growth rate or final cell density. Thus it is hematin and not the growth rate that causes slime production.

Figure 8. Comparison of growth for a non-adherent staphylococcal isolate, 9134 in TSB, NNS, and NH.

2. Comparison of growth for a non-adherent clinical isolate, 9134 in TSB, NNH, and NNS

3. Comparison of growth for an adherent clinical isolate, 8894 in TSB, NNH, and NNS

4. Comparison of growth for *S. epidermidis* RP62A, an adherent positive control

5. Comparison of growth for *S. epidermidis* RP62A, positive reference, in NNH plus lipid and NNS plus lipid

6. Comparison of growth for non-adherent clinical isolate, 9134, in NNH plus lipid and NNS plus lipid.

7. Comparison of growth for adherent positive clinical isolate, 8894, in NNH plus lipid and NNS plus lipid.

10. Comparison of non-adherent clinical isolate 9134 in TSB.
U broth and hematin supplemented broth.

Figure 9. Growth of *S. hominis*, and *S. hemolyticus* in NNS.

Figure 8. Growth of ATCC coagulase-negative staphylococci in TSB; *S. hominis*, *S. xylosus*, *S. cohnii*, *S. simulans*, *S. sciuri*, *S. hemolyticus*, and *S. epidermidis*.

DISCUSSION

Several investigators have shown that some strains of coagulase-negative staphylococci produce an extracellular material or adherent material. The ability to support adherent growth is a stable genetic characteristics and did not change in the repeated laboratory transfers involved with this research. However, We did observe that some strains have a greater capacity to produce adherent growth than other strains do. The ability for a specific strain to form adherent biofilm was determined by the growth medium. Diebel and Jones (1962) worked with carbon sources that would support anaerobic growth of S. epidermidis. Using a semi-synthetic medium, they observed that several carbon sources would support anaerobic growth but only glucose gave slimy cultures. We also have found that adherent growth was obtained only when glucose was the carbon and energy source.

Christensen (1982) has reported that adherent growth of S. epidermidis was obtained in TSB only when a low concentration of glucose was used. However, the neonatal nutrients administered in the catheters contain 13% glucose and this probably results in a high concentration of glucose at the blood vessel-catheter junction. If adherent growth is a virulence factor, it would be expected that all coagulase-negative clinical isolates would produce adherent growth. In this study, adherent biofilm from the clinical isolates was only

obtained with the coagulase-negative isolates that keyed out to be S. epidermidis or S. saprophyticus.

Thus we wanted to find a semi-synthetic medium that would be more physiologically oriented than TSB. The "U" medium supported good growth of all clinical isolates. However, no adherent growth could be demonstrated with the known adherent strains, RP62A or any of our clinical isolates. The addition of hematin to the "U" medium did show adherent growth which was comparable to that obtained when the strains were grown in TSB. Thus it would appear that physiological concentrations of hematin or some blood factors may facilitate adherent growth.

A similar finding was obtained when the strains were tested for phenotypic variation. Adherent strains were compared for the ability to exhibit phenotypic variation when grown on: 1) the high salt-low glucose agar (Memphis agar) of Christensen; 2) the semi-synthetic low-glucose "U" medium; and 3) hematin supplemented "U" medium. In contrast to adherence promotion, the addition of hematin to "U" medium did not affect colony morphology. No correlation of the ability to produce colony morphotypes could be seen between Memphis agar and "U" agar for any strain tested.

Since growth in blood would include the presence of serum, we looked at the ability of serum to increase the growth rate of several clinical isolates. Neonatal nutrient were used as a medium base in these studies since this would be the major source of nutrients under the catheterization procedures used for neonates. The growth rate

was the same for a given strain when the neonatal nutrients were diluted with water or fetal calf serum to give 6.5% glucose. However, the final growth yield was always higher when serum had been added. No adherent growth under these conditions was observed even with the adherent-positive strain, RP62A and adherent clinical isolates unless hematin was present. The addition of hematin to the neonatal nutrients culture resulted in adherent growth when the cultures were incubated under static conditions.

Generally the neonatal catheterization is performed with a lipid supplement to the neonatal nutrient. The addition of lipid to the growth medium did not change the growth rate of any strain tested. The adherent biofilm was again obtained with the addition of hematin and incubation of the cultures under static condition. The amount of hematin that stimulated adherent growth was consistent with the concentration of hematin calculated to be present in adult serum. When NNS or NNH cultures with or without lipid were incubated with shaking, there was no adherent growth under any condition tested. However, there was a heavy ring of growth at the air-medium interface of all cultures that contained neonatal nutrients. There was no interface growth when the strains were grown in TSB. Cultures grown in "U" medium did give a slight adherent ring at the interface and the cells were flocculated in the medium during incubation with shaking.

Clinical isolates obtained initially from the catheters often gave coagulase-negative staphylococci a few days prior to obtaining a

positive blood culture from the neonates. This could result in adherent growth on the irregularities of the catheter walls before cultures were sloughed into the blood stream. A comparison of growth rates using neonatal nutrients diluted 1:1 with water or fetal calf serum showed no difference in the growth rate. Russel et al (1987) observed that clinical isolates of S. epidermidis adhered to rough areas of heparin treated catheters and that the adherent growth of S. epidermidis was increased when fibronectin was present in the incubated medium. The neonatal nutrients used contained heparin. Thus fibronectin coming from the serum in addition to the presence of hematin could give an ideal condition for adherent growth.

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