A chemically defined medium for slime production by coagulase-negative staphylococci

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Summary. In a chemically defined medium in which the principal constituents were glucose, 18 amino acids, two purines and six vitamins, eight strains of coagulase-negative staphylococci grew as rapidly and heavily as in tryptic soy broth. Slime formation was slightly better in the defined medium than in tryptic soy broth.

Introduction

In recent years coagulase-negative staphylococci (CNS) have come to be recognised as a major cause of septicaemia in patients with implanted prosthetic devices and intravenous catheters. Some isolates produce extracellular slime which is thought to be important in the genesis of these plastic-associated infections.^{1–5} The slime substance is loosely bound to staphylococcal cells and can be washed off.⁶

In published methods of preparing staphylococcal slime, bacteria have been grown under fluid on complex media solidified with agar^{7–9} or in liquid culture,¹⁰ and, after separating the slime layer and removing bacteria by centrifugation, the supernatant liquid has been used as starting material for analyses. These slime preparations inevitably contain some ingredients of high molecular weight from the media. To avoid this problem, Peters *et al.*⁶ used an elaborate chemically defined medium formulated to meet the growth requirements of streptococci;¹¹ it contained ingredients not usually required by staphylococci. The present study was designed to devise a simpler chemically defined medium that would support growth and maximal slime production by CNS.

Materials and methods

Bacterial strains

Eight strains of *Staphylococcus epidermidis* were used: reference strains RP-12 (ATCC 35983) and RP-62A (ATCC 35984); clinical isolates C762, C1543, C1276 and A2057, all isolated in the Bacteriology Department, Royal Hallamshire Hospital, Sheffield from infected long intravenous catheters; and skin isolates C362 and C988. Strains RP-12, RP-62A, C762

and C1543 produced slime, as determined by the method of Christensen *et al.*³

Growth requirements

Amino acid requirements for growth were determined by growth tests with Gladstone's medium.¹² Each amino acid was prepared as a concentrated stock solution so that the addition of 0.2 ml of each gave the final concentration required in 10 ml of the complete medium. Bacteria were grown overnight on nutrient-agar slopes, washed twice in physiological saline and resuspended in saline to a concentration of *c.* 1×10^8 cfu/ml; 0.1 ml of this suspension was added to 10 ml of medium in a 15 × 1.7-cm test tube and incubated with shaking at 37°C. Growth was estimated by eye after incubation for 24 and 48 h.

Carbohydrate requirements were determined with the same medium but replacing glucose by different sugars (galactose, mannose, fructose, sucrose, lactose and maltose), each 1% w/v. The experimental procedures (inoculation, incubation and growth estimation) were as described for the determination of amino acid needs.

The effect of various supplements on slime production was investigated by adding them to vitamin-containing medium A¹³—Na₂HPO₄·12H₂O, 11.2 g; KH₂PO₄, 2.4 g; (NH₄)₂SO₄, 2 g; MgSO₄·7H₂O, 50 mg; MnCl₂·4H₂O, 4 mg; FeSO₄·7H₂O, 2.8 mg; glucose, 10 g; biotin, 0.1 mg; nicotinic acid, 2 mg; D-pantothenic acid, Ca salt, 2 mg; pyridoxal, 4 mg; pyridoxamine dihydrochloride, 4 mg; riboflavin, 2 mg; thiamin hydrochloride, 2 mg; and water to 1 L. Medium A plus vitamins was prepared by mixing four separately autoclaved solutions. **Solution A:** Na₂HPO₄·12H₂O, KH₂PO₄, (NH₄)₂SO₄ at × 5 final concentration; **Solution B:** salts (Mg²⁺, Mn²⁺ and Fe²⁺) dissolved together at × 50 final concentration in 0.01 M, H₂SO₄; **Solution C:** glucose 20% w/v; **Solution D:** a mixture of vitamins prepared at × 100 final concentration. For 500 ml of medium A, 100 ml of solution A was autoclaved with 360 ml of glass-

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distilled water and, after cooling, 10 ml of solution B, 25 ml of solution C and 5 ml of solution D were added.

The supplements were: 1. Na acetate·3H₂O 0.5% w/v. 2. Trace elements (/L of medium), 5 ml of stock solution containing FeSO₄(NH)₂SO₄·6H₂O, 116 mg; HBO₃, 232 mg; CoSO₄·7H₂O, 95.6 mg; CuSO₄·5H₂O, 8 mg; MnSO₄·4H₂O, 8 mg; (NH₄)₆Mo₇O₂₄·4H₂O, 22 mg; ZnSO₄·7H₂O, 174 mg; 1N H₂SO₄, 10 ml; water to 1 L. 3. Casamino acids (Difco) 3% w/v. 4. Yeast extract (Oxoid) 0.1% w/v. 5. Purines/pyrimidines, 20 mg each of adenine, guanine and uracil /L of medium. 6. Defined amino acid mixture containing (mg/L): L-aspartic acid, 150; L-alanine, 100; L-arginine, 100; L-cystine, 50; glycine, 100; L-glutamic acid, 150; L-histidine, 100; L-isoleucine, 150; L-lysine, 100; L-leucine, 150; L-methionine, 100; L-phenylalanine, 100; L-proline, 150; L-serine, 100; L-threonine, 150; L-tryptophan, 100; L-tyrosine, 100; L-valine, 150.

Preparation of the chemically defined medium

The ingredients of the medium were first made up separately in five groups (table I). The components of group 1 were dissolved in 700 ml of water. Groups 3, 4 and 5 were made as concentrated solutions as follows: group 3, ×100; group 4, ×20 and group 5, ×100. Except for group 2, the groups were mixed, made up to 900 ml with glass-distilled water and autoclaved at 121°C for 15 min. Group 2 ingredients (made up to 100 ml in glass-distilled water) were autoclaved separately and added to the rest when cool. For brevity the defined medium is referred to as medium HHW (Hussain, Hastings, White).

Detection of slime production

Because there is at present no single generally

Table I. Composition of a new chemically-defined medium HHW for slime production by CNS

Ingredients	mg/L	Ingredients	mg/L
Group 1		Group 2	
Na ₂ HPO ₄ ·2H ₂ O	10 000	Glucose	10 000
KH ₂ PO ₄	3000	MgSO ₄ ·7H ₂ O	500
L-Aspartic acid	150		
L-Alanine	100	Group 3	
L-Arginine	100	Biotin	0.1
L-Cystine	50	Nicotinic acid	2
Glycine	100	D-Pantothenic acid, Ca salt	2
L-Glutamic acid	150	Pyridoxal	4
L-Histidine	100	Pyridoxamine dihydrochloride	4
L-Isoleucine	150	Riboflavin	2
L-Lysine	100	Thiamin hydrochloride	2
L-Leucine	150		
L-Methionine	100	Group 4	
L-Phenylalanine	100	Adenine sulphate	20
L-Proline	150	Guanine hydrochloride	20
L-Serine	100		
L-Threonine	150	Group 5	
L-Tryptophan	100	CaCl ₂ ·6H ₂ O	10
L-Tyrosine	100	MnSO ₄	5
L-Valine	150	(NH ₄) ₂ SO ₄ ·FeSO ₄ ·6H ₂ O	6

(Final pH value 7.2)

accepted method for determining slime production, three different methods were compared: a qualitative tube assay; a spectrophotometric method that assessed biofilm on the bottom of wells but not on their walls; and an ATP bioluminescence method that measured the biomass of all adherent bacteria, whether they were on the bottom of the wells or on their walls.

Tube method.³ The test medium (10 ml) in a glass 28-ml screw-capped bottle was inoculated with 0.1 ml of a saline suspension of bacteria of OD₅₄₀ 1.0 and containing *c.* 1 × 10⁹ cfu/ml. After incubation for 18 h at 37°C, the bottle contents were poured out and 10 ml of an aqueous solution of alcian blue 0.1% w/v was added to stain adherent bacteria. A blue-stained film lining the bottle walls was taken to indicate slime production.

ATP bioluminescence assay. Cultures grown overnight in HHW medium or tryptic soy broth were diluted 1 in 100 with fresh medium to contain *c.* 1 × 10⁷ cfu/ml and 200-μl volumes were poured (in triplicate) into flat-bottomed wells of a polystyrene microtitration plate (Amersham International plc, Buckinghamshire). The plate was covered and incubated at 37°C for 18 h. The fluid in the wells was aspirated and the wells were washed twice with saline by means of an automated plate washer (Amersham International). ATP was extracted from adherent bacteria by adding 200 μl of trichloroacetic acid 2.5% w/v. A 10-μl sample of the extract was then transferred to a well of another microtitration plate containing 200 μl of Tris-HCl buffer (pH 7.8); 30 μl of ATP monitoring reagent (LKB-Wallac Oy, Finland, 1243-102; one vial reconstituted with 10 ml of distilled water) was added and light output was measured in a microtitration plate luminometer (Amerlite, Amersham International). A second reading was made after adding 10 μl of ATP standard (LKB-Wallac Oy, Finland, 1243-201; one vial reconstituted with 10 ml of distilled water). The light reading in mV was converted to picomoles of ATP according to the following formula: ATP(pmoles) = [Light output (mV) from sample / Light output (mV) from standard] × Amount of standard added (picomoles).

Spectrophotometric method. The method of Christensen *et al.*¹⁴ was used with three replicates. Bacterial suspensions (200 μl) in appropriate medium containing *c.* 1 × 10⁷ cfu/ml were poured in triplicate into a polystyrene 96-well flat-bottomed plate. After incubation for 18 h at 37°C, fluid in the wells was aspirated and the wells were washed twice with saline. Adherent growth was stained with safranin 0.1% for 1 min, washed with distilled water and allowed to dry. OD was determined at 490 nm with a Microelisa Mini-reader MR590 (Dynatech, Guernsey, Channel Islands).

Measurement of growth

Medium HHW and tryptic soy broth (100 ml of each in separate 250-ml flasks) were each inoculated

with 0.1 ml of a 1 in 10 dilution of an overnight culture in the same medium and incubated with shaking at 37°C for 24 h. Turbidity was measured in an EEL colorimeter (Evans Electroselenium Co. Ltd, Halstead, Essex); the zero was adjusted with uninoculated medium. Growth curves were made from viable counts on blood agar after growing the strains in these two media.

Results

Measurement of growth

Growth curves constructed from viable counts made with strain RP-62A growing in medium HHW and tryptic soy broth are shown in the figure. Growth was slow at first in the medium HHW, but after 18 h the numbers of viable bacteria (cfu) in both media were the same. Similar results were obtained with the other strains.

Growth requirements

All strains required valine and arginine for growth, as described by White and Woods.¹⁵ Leucine and isoleucine were required by all strains for growth by 18 h; their absence delayed growth. One strain only did not require tyrosine and two only did not need threonine for growth in 18 h. No strain required aspartic acid and two strains only required proline and histidine. However, if more than two of the amino acids in table II were omitted simultaneously, none of the strains grew. There were no clear differences in amino-acid requirements of slime-producing and slime

Figure. Growth of strain RP-62A in medium HHW (○) and in tryptic soy broth (●) with shaking at 37°C.

non-producing isolates; the two slime-producing reference strains, RP-62A and RP-12, had identical amino-acid requirements (table II).

Three slime-producing strains (RP-12, RP-62A and C762) and one slime non-producing strain (C988) grew well with all carbohydrate sources tested (table III). Strains C1543, A2057, C362 and C1276 grew well with glucose, but either grew poorly or not at all with most of the other sugars. There was no clear difference in the carbohydrate requirements of slime-producing and slime non-producing strains. Glucose was the only

Table II. Amino acid needs of eight strains of CNS

Omission from medium of Gladstone ¹²	Growth of strains							
	slime non-producers				slime producers			
	A2057	C988	C362	C1276	C1543	C762	RP-12	RP-62A
Alanine	g	g	d	d	—	g	g	g
Arginine	—	—	—	—	—	—	—	—
Aspartic acid	g	g	g	g	g	g	g	g
Cysteine	g	g	—	—	d	—	—	—
Glutamic acid	—	—	—	—	—	g	g	g
Glycine	g	g	g	d	d	d	g	g
Histidine	g	g	—	d	g	g	g	g
Isoleucine	—	d	d	—	d	—	—	d
Leucine	—	—	—	—	—	d	d	d
Lysine	g	g	g	—	d	d	g	g
Methionine	g	g	g	g	g	d	g	g
Phenylalanine	g	—	d	—	g	d	g	g
Proline	g	—	g	g	g	—	g	g
Serine	g	g	g	d	d	g	g	g
Threonine	g	—	d	g	—	—	d	d
Tryptophan	g	g	g	g	g	—	—	—
Tyrosine	—	g	—	—	—	—	d	d
Valine	—	—	—	—	—	—	—	—

g, growth when amino acid omitted; d, growth delayed (more than 18 h); —, no growth when amino acid omitted. (All amino acids except glycine were L-isomers).

Table III. Carbohydrate needs for growth by CNS

Carbohydrate source* in the medium of Gladstone ^{1,2}	Growth of strains							
	slime non-producers				slime producers			
	A2057	C988	C362	C1276	C1543	C762	RP-12	RP-62A
None	—	—	—	—	—	—	—	—
Glucose	g	g	g	g	g	g	g	g
Galactose	d	g	d	—	—	g	g	g
Mannose	d	g	d	—	—	—	g	g
Fructose	d	g	d	d	g	g	g	g
Sucrose	g	g	d	d	g	g	g	g
Lactose	d	g	d	g	d	g	g	g
Maltose	d	g	—	g	—	g	g	g

—, No growth; g, growth; d, growth delayed.

* All at 1% w/v in medium.

carbohydrate source acceptable to all the strains tested, and in its presence growth was maximal.

Requirements for slime production

Slime-producing strains (RP-12, RP-62A, C1543 and C762) were inoculated into medium A after addition of various supplements at differing concentrations and in various combinations. The results obtained are summarised in table IV. Vitamins and an organic nitrogen source were always required for growth. Adding yeast extract promoted slime production; supplementing with Casamino acids alone allowed growth but the addition of trace elements, or adenine and guanine, or sodium acetate, was required for slime production. Replacing yeast extract or Casamino acids by a defined amino-acid mixture formulated in relation to the previous growth-promotion experiments (table II) allowed excellent slime elaboration by all four test strains, as long as either trace elements or adenine and guanine were present.

Table IV. Slime production by CNS in supplemented medium A

Supplements to medium A + vitamins	Growth, and slime production, of strains			
	RP-12	RP-62A	C1543	C762
Nil	—	—	—	—
Na acetate · 3H ₂ O	—	—	—	—
Trace elements	—	—	—	—
CA 3%	g	g	g	g
YE 0.1%	S	S	S	S
CA 3% + trace elements	S	S	S	S
CA 3% + adenine/guanine	S	S	S	S
CA 3% + uracil	g	g	g	g
CA 3% + Na acetate · 3H ₂ O	S	S	S	S
DAAM	g	g	g	g
DAAM + trace elements	S	S	S	S
DAAM + adenine/guanine	S	S	S	S
DAAM + Na acetate · 3H ₂ O	S	g	g	g

—, No growth; g, growth; S, growth + slime production (assay by tube method). CA, Casamino acids (Difco); YE, yeast extract (Oxoid); DAAM, defined amino-acid mixture.

This formulation is medium HHW and is shown in detail in table I.

Medium HHW and tryptic soy broth (a medium used widely by other workers) were compared for their ability to facilitate slime production by CNS. Using the tube method, it was hard to detect differences between the two media with good slime producers (strains RP-62A and RP-12) but the two weak slime producers (strains C1543 and C762) produced slime more readily in medium HHW (table V).

When measured spectrophotometrically, slime production was better in medium HHW (table VI). Similar results were obtained when the adherent biomass was measured by ATP bioluminescence (table VII).

Table V. Assay of slime production by tube test

Strain no	Slime production in	
	medium HHW	tryptic soy broth
R62A	+++	+++
RP-12	++	++
C762	++	+
C1453	++	+
C362	—	—
C988	—	—

—, No slime, + — + + +, increasing intensity of colour and thickness of stained adherent biomass, as judged by two observers.

Table VI. Assay of slime by spectrophotometric method

Strain no.	Mean *(SD) OD ₄₉₀ after growth in	
	medium HHW	tryptic soy broth
RP-62A	1.79 (0.19)	1.09 (0.11)
RP-12	0.41 (0.13)	0.23 (0.09)
C762	0.30 (0.07)	0.12 (0.10)
C1543	0.34 (0.10)	0.14 (0.08)
C362	0.05 (0.01)	0.06 (0.02)
C988	0.05 (0.01)	0.05 (0.01)

* Mean of results of nine tests.

Table VII. Assay of slime by measurement of ATP bioluminescence

Strain no.	Mean *(SD) picomoles of ATP after growth in	
	medium HHW	tryptic soy broth
RP-62A	68.65 (4.27)	51.12 (3.90)
RP-12	50.4 (4.12)	31.89 (3.1)
C762	32.3 (6.2)	19.4 (4.9)
C1543	37.1 (5.8)	21.6 (4.6)
C362	17.91 (1.19)	17.1 (1.10)
C988	19.88 (2.5)	7.19 (6.63)

* Mean of results of nine tests.

Discussion

There are a few reports of the chemical nature of slime produced by staphylococci and, in almost all studies, complex media were used to prepare it.⁷⁻¹⁰

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The use of complex media to isolate slime present in the culture fluid is disadvantageous because high molecular weight ingredients from the medium are likely to be present. Peters *et al.*⁶ used a chemically defined medium but the effects and requirements of different components on growth and slime production were not recorded. The newly developed simpler chemically defined medium described here (HHW) contains components essential for growth or slime production only and, when tested with several strains and methods, appears to be at least as good as tryptic soy broth for supporting growth and slime production.

The exact chemical nature of slime is not known⁶ and there is no specific marker for its detection. Medium HHW is now being used to collect slime material from CNS for analysis.

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