Growth Requirements of Some Small-Colony-Forming Variants of *Staphylococcus aureus*

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Nine stable, auxotrophic, small-colony-forming variants of *Staphylococcus aureus* were isolated. They were of two types. One type, from broth cultures containing varying concentrations of gentamicin or kanamycin, required heme for normal growth. The second type, from the blood culture of a patient who had been treated with penicillin and oxacillin, required long-chain unsaturated fatty acids for normal growth. These small-colony-forming variants differed from their parents with respect to specific metabolic activities, many of which may be attributed to an impaired electron transport system and related functions. A laboratory procedure for detecting possible small-colony variants in clinical specimens was proposed.

Since small-colony-forming variants (SCV) were first described in 1911 by Kolle and Hetsch (13), much about their morphology, metabolism, and physiology has been conjecture. SCV have since been reported in various species of bacteria and yeasts (2, 19).

Isolation of primary pure cultures of SCV from clinical material was a rarity, partly due to the difficulty in obtaining suitable culture media (7) and to the increased incubation time needed for the SCV to appear in conventional culture media (4). In many cases SCV appeared in patients undergoing antibiotic therapy (5, 7, 8, 15, 20). These antibiotics may have provided conditions *in vivo* and *in vitro* that were suboptimal for the growth of the normal cells, thus selecting for SCV. *In vitro* studies indicated that agents other than antibiotics can select for SCV (8) and, further, that SCV can arise spontaneously (15).

Studies were undertaken on the presumption that many SCV were nutritionally deficient. They were selected or had developed as a result of growth in the presence of inhibitory agents (20). Among the substances found to be cardinal growth-stimulating requirements for the SCV, and thus causing SCV to form phenotypic revertants, were antibiotics (1), amino acids (5, 8), vitamins (18), hemin (6, 11, 20), long-chain, unsaturated fatty acids (2), CO₂ (15, 16), carbohydrates (19), and thioglycolate (5).

The present study was undertaken (i) to isolate SCV of *S. aureus*, (ii) to determine the growth-stimulating requirements of the SCV and the location of the metabolic block, (iii) to define the implications and significance of SCV

in microbial persistence, (iv) to define the problems of detecting SCV in laboratories, and (v) to make recommendations for the culturing of specimens when SCV are suspected.

MATERIALS AND METHODS

Bacterial strains. Four strains of *S. aureus* were used. Three strains were derived from American Type Culture Collection (ATCC) cultures that were obtained from the stock cultures from the Department of Medical Microbiology, University of Nebraska Medical Center, and were designated as strains K, M, and N. The fourth strain, obtained from the Diagnostic Bacteriology Laboratory, University of Nebraska Medical Center, was isolated from a blood culture and was designated as strain C. These four strains (Table 1) were purified by cloning on Trypticase soy agar (TSA) (BBL) plates through six successive transfers and stored at 4°C on TSA slants.

Isolation of SCV. A modification of the Wise and Spink (19) method was used to isolate SCV from the ATCC parent strains. Each parent was inoculated into 5 ml of Trypticase soy broth (TSB) (BBL). After incubation at 37°C for 24 h, 0.1 ml of the following antibiotics was added at a final concentration of 10, 100, and 1,000 µg/ml: clindamycin HCl hydrate (Upjohn), erythromycin (Abbott Laboratories), gentamicin sulfate (Schering Laboratories), and kanamycin sulfate (Bristol Laboratories). Incubation was continued for 1 to 2 weeks at 37°C, during which time a portion was removed and streaked for isolation. These plates were incubated for 1 to 7 days at 37°C and examined with a dissecting microscope for the presence of small colonies. Small colonies were serially subcultured until their stability was determined. The C₁ SCV was isolated, with difficulty, as a primary laboratory isolate from the parent strain C, which was obtained from a blood culture of a 4-

TABLE 1. Source and strains of *S. aureus* and concentration of antibiotics used for the isolation of SCV

Strain ^a	Source	Concn of antibiotic
Parent K	ATCC 6538P	
K ₁		100 µg of gentamicin per ml
K ₄		100 µg of kanamycin per ml
K ₅		1,000 µg of kanamycin per ml
Parent M	ATCC 11988	
M ₂		1,000 µg of kanamycin per ml
Parent N	ATCC 15752	
N ₁		10 µg of gentamicin per ml
N ₄		100 µg of kanamycin per ml
N ₅		1,000 µg of kanamycin per ml
N ₆		1,000 µg of gentamicin per ml
Parent C ^b	Primary laboratory isolate	
C ₁	Primary laboratory isolate	

^a Subscripts represent SCV.^b Patient was previously treated with penicillin and oxacillin.

week-old male, who, approximately 1 week prior to hospital admission for osteomyelitis, had been treated with penicillin and exacillin. All of the stable SCV were grown on TSA slants for 48 h at 37°C and stored, as were the parents.

Preparation of growth-stimulating factors. Yeast extract (Antibiotic Laboratory, University of Nebraska Medical Center) and Tween 80 (Fisher Scientific Corp.) were prepared as 10% (vol/vol) aqueous solutions and sterilized by autoclaving. Sterile casein hydrolysate (Nutritional Biochemical Corp.), sterile dithiothreitol as present in Sputolysin (Calbiochem), and heme in the form of X-factor strips (BBL) were used as commercially prepared. Human plasma and human hemolyzed blood were prepared from whole blood treated with ethylenediaminetetraacetic acid. The porphyrin precursors, δ-aminolevulinic acid HCl, porphobilinogen, coproporphyrin III tetraethyl ester, protoporphyrin-IX-dimethyl ester, and hemein (Sigma Chemical Co.), were prepared according to Devriese (6). The stearic acid (J. T. Baker Chemical Co.) was prepared as a 10% (wt/vol) mixture in 95% ethanol. This mixture was slowly heated until a solution was obtained, but had to be reheated to solution before use. The unsaturated fatty acids, oleic acid (Nu Chek Prep), linoleic acid, and linolenic acid (Delta Chemical Works), were prepared as a 10% (vol/vol) solution in 95% ethanol. All solutions were stored at 4°C.

Screening growth-stimulating factors. A dilute

suspension of each SCV was made in TSB and streaked onto TSA plates with a swab. Filter-paper disks were impregnated with 0.1 ml of each of the growth-stimulating factors. After allowing the surface of the plates to air dry, the impregnated disks were aseptically placed onto the surface of the plates. The plates were incubated for 1 to 3 days at 37°C. Stimulation of the SCV was noted by an increase in pigmentation and colonial size on an area around the disk. Parental strains were included as controls.

Cultural characterization. SCV were measured with a low-power dissecting microscope with a calibrated ocular reticule. Occasionally, larger colonies, greater than 1 mm in diameter, were measured with a metric rule. Pigmentation was observed macroscopically and characterized by visual inspection of full-grown colonies.

Biochemical characterization. Hemolysis was observed on 5% sheep blood agar (Difco) plates, coagulase production was determined (10), and catalase production was measured by using 3% hydrogen peroxide. The ability to reduce 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co.) was determined by the procedure of Ogur et al. (14).

Antibiotic susceptibility testing. Antibiotic studies were done by a modification of the Bauer-Kirby (3) method. Suspensions of the strains, prepared in brain heart infusion broth (Difco), were adjusted to an optical density reading of 1.034 for strains K and M and their SCV, and an optical density of 1.022 for strains N and C and their SCV on a Coleman Junior II model 6/20 spectrophotometer at a wavelength of 550 nm. A 0.01-ml amount of each suspension was transferred to 8 ml of 0.3% soft agar at 55°C and overlaid onto Mueller-Hinton agar (Difco) plates. Sensi-discs (BBL) were dispensed onto the surface of the plates. After 24 h of incubation at 37°C the plates were read and zone sizes were recorded in millimeters.

RESULTS

SCV. Nine stable SCV were isolated from broth cultures containing varying concentrations of antibiotics and designated K₁, K₄, K₅, M₂, N₁, N₄, N₅, N₆, and C₁ (Table 1).

Growth-stimulating experiments. Two types of SCV were observed. All SCV of K and M and three N variants were stimulated by hemolyzed blood, heme in the form of X-factor, and hemein. None of the variants was stimulated by any of the hemein precursors assayed (Table 3). The C₁ variant was stimulated by Tween 80, oleic acid, linoleic acid, and linolenic acid. However, stearic acid was not stimulatory (Table 4). Yeast extract, casein hydrolysate, dithiothreitol, and human plasma did not enhance the growth of any of the SCV (Table 2).

Cultural and biochemical characteristics. The size of the parent colonies ranged from 2 to 3 mm in diameter, whereas the SCV ranged from 0.01 to 0.1 mm in diameter. The pigmenta-

TABLE 2. Preliminary screening for potential growth-stimulating factors for parents and SCV of *S. aureus*

Strain ^a	Growth-stimulating effect						
	Yeast extract	Tween 80	Casein hydrolysate	Dithiothreitol	Human plasma	Hemolyzed blood	X-factor
K	— ^b	—	—	—	—	—	—
K ₁	—	—	—	—	—	+	+
K ₄	—	—	—	—	—	+	+
K ₅	—	—	—	—	—	+	+
M	—	—	—	—	—	—	—
M ₂	—	—	—	—	—	+	+
N	—	—	—	—	—	—	—
N ₁	—	—	—	—	—	—	—
N ₄	—	—	—	—	—	+	+
N ₅	—	—	—	—	—	+	+
N ₆	—	—	—	—	—	+	+
C	—	—	—	—	—	—	—
C ₁	—	+	—	—	—	—	—

^a Subscripts represent SCV.^b —, No stimulation; +, stimulation.TABLE 3. Growth-stimulating effects of hemolyzed blood and porphyrin intermediates for parents and SCV of *S. aureus*

Strain ^a	Growth-stimulating effect						
	Hemin	X-factor	Hemolyzed blood	Δ ALA	Porphobilinogen	Coproporphyrin	Protoporphyrin III (no. IX)
K	— ^b	—	—	—	—	—	—
K ₁	+	+	—	—	—	—	—
K ₄	+	+	+	—	—	—	—
K ₅	+	+	+	—	—	—	—
M	—	—	—	—	—	—	—
M ₂	+	+	+	—	—	—	—
N	—	—	—	—	—	—	—
N ₁	—	—	—	—	—	—	—
N ₄	+	+	+	—	—	—	—
N ₅	+	+	+	—	—	—	—
N ₆	+	+	+	—	—	—	—
C	—	—	—	—	—	—	—
C ₁	—	—	—	—	—	—	—

^a Subscripts represent SCV.^b —, No stimulation; +, stimulation.

tion of the parent colonies was a typical golden. All of the variants except C₁ formed nonpigmented colonies. The C₁ developed cream-colored colonies. All of the SCV, like their parents, were β-hemolytic and catalase and 2,3,5-triphenyltetrazolium chloride positive. Except for the N₄ variant, all were coagulase positive (Table 5).

Antibiotic susceptibilities. Although the zone sizes varied, the parent strains K and M showed

identical susceptibility patterns with their respective SCV. Two SCV of the N strain exhibited resistance to chloramphenicol, whereas all of the N variants were intermediately sensitive to erythromycin and sensitive to penicillin G. C₁ was resistant to erythromycin (Table 6).

DISCUSSION

Most SCV of *S. aureus* were isolated from broth cultures containing a concentration of

antibiotics inhibitory to the parent strains. The antibiotics used for selection apparently killed or altered the actively metabolizing cells, thus selecting for SCV.

The heme requirements for some of the SCV have previously been shown to be related to a defect in the electron transport system (ETS). Tien and White (17) demonstrated that SCV form a functional ETS, albeit slowly. Joyce and White (12) showed that by inhibiting the formation of the ETS, various metabolic activities

were depressed, specifically the synthesis of the cytochromes, lipids, and carotenoids from the isoprenoid lipids. Jensen (11) found that in the absence of heme, SCV were completely devoid of hematin compounds, thus resulting in an impaired ETS and, therefore, in energy metabolism. It is possible that the heme-requiring SCV isolated in this experiment may belong to this class of organisms with an impaired ETS. Since these SCV were stimulated by heme but not by any porphyrin precursors, it appeared that the metabolic block was between the proto-porphyrin and the heme. This suggests that the SCV cannot insert the ferric iron-porphyrin into the pyrrole structure because of a defect in the heme synthetase. This represents a rather specific mechanism since the ferrous iron-porphyrin can be inserted, as is evidenced by the fact that catalase is synthesized (Table 5).

The clinical SCV, strain C₁, may have a defective lipid synthesis and, therefore, utilize only unsaturated fatty acids. There may also be an accompanying impaired ETS. Lack of stimulation by the yeast extract suggested that the defect was not a lack of biotin, which was needed for lipid synthesis, but rather in the dehydrogenase needed to desaturate the saturated fatty acid.

The reduced pigmentation observed in all of the SCV might be correlated with the defective ETS. A depression of the synthesis of isoprenoid lipid due to the decreased amount of energy from the impaired ETS caused a decrease in the synthesis of the carotenoids that were needed for pigment production.

TABLE 4. Growth-stimulating effects of fatty acids for parents and SCV of *S. aureus*

Strain ^a	Growth-stimulating effect				
	Tween 80	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
K	—	—	—	—	—
K ₁	—	—	—	—	—
K ₄	—	—	—	—	—
K ₅	—	—	—	—	—
M	—	—	—	—	—
M ₂	—	—	—	—	—
N	—	—	—	—	—
N ₁	—	—	—	—	—
N ₄	—	—	—	—	—
N ₅	—	—	—	—	—
N ₆	—	—	—	—	—
C	—	—	—	—	—
C ₁	+	—	+	+	+

^a Subscripts represent SCV.

^b —, No stimulation; +, stimulation.

TABLE 5. Biochemical and cultural properties of parents and SCV of *S. aureus*

Strain ^a	Biochemical and cultural properties					
	Diam (mm)	Pigment ^b	Hemolysis	Coagulase	Catalase	Tetrazolium reduction
K	2	Y	β	+	+	+
K ₁	0.01-0.1	N	β	+	+	+
K ₄	0.01-0.1	N	β	+	+	+
K ₅	0.01-0.1	N	β	+	+	+
M	2	Y	β	+	+	+
M ₂	0.01-0.1	N	β	+	+	+
N	3	Y	β	+	+	+
N ₁	0.01-0.1	N	β	+	+	+
N ₄	0.01-0.1	N	β	—	+	+
N ₅	0.01-0.1	N	β	+	+	+
N ₆	0.01-0.1	N	β	+	+	+
C	2	Y	β	+	+	+
C ₁	0.01-0.1	C	β	+	+	+

^a Subscripts represent SCV.

^b Y, Yellow-orange to golden; C, cream; N, nonpigmented.

TABLE 6. Antibiotic susceptibilities of parents and SCV of *S. aureus*

Strain ^a	Zone size (mm) ^b											
	Tetra ^c	Chlor	Kana	Ampi	Cephalo	Erythro	Linco	Oxa	Meth	Pen G	Clinda	Naf
K	S	S	S	S	S	S	S	S	S	S	S	S
	22	30	28	40	32	32	33	32	32	42	32	32
K ₁	S	S	S	S	S	S	S	S	S	S	S	S
	42	36	20	40	46	40	32	34	32	46	40	32
K ₄	S	S	S	S	S	S	S	S	S	S	S	S
	38	36	20	40	40	40	36	32	32	42	40	32
K ₅	S	S	S	S	S	S	S	S	S	S	S	S
	36	32	20	40	46	40	36	34	32	46	42	32
M	S	S	S	S	S	S	S	S	S	S	S	S
	38	30	32	40	30	30	32	28	28	40	30	28
M ₂	S	S	S	S	S	S	S	S	S	S	S	S
	38	32	30	40	40	40	38	32	30	46	42	32
N	R	S	S	I	S	R	S	S	S	R	S	S
	12	30	30	20	36	13	30	28	28	16	28	28
N ₁	R	R	S	I	S	I	S	S	S	S	S	S
	12	12	20	26	40	16	28	30	34	24	40	32
N ₄	R	R	S	I	S	I	S	S	S	S	S	S
	12	12	20	26	40	16	28	30	34	24	40	32
N ₅	R	S	S	I	S	I	S	S	S	S	S	S
	12	38	20	26	40	14	28	30	36	22	40	32
N ₆	R	S	S	I	S	I	S	S	S	S	S	S
	12	38	20	26	40	14	28	30	36	22	40	32
C	S	S	S	R	S	I	S	S	S	R	S	S
	38	28	28	18	30	14	30	22	22	14	30	22
C ₁	S	S	S	R	S	R	S	S	S	R	S	S
	30	30	18	0	24	12	22	18	20	0	30	20

^a Subscripts represent SCV.^b S, Sensitive; I, intermediate; R, resistant.^c Tetra, tetracycline; Chlor, chloramphenicol; Kana, kanamycin; Amp, ampicillin; Cephalo, cephalothin; Erythro, erythromycin; Linco, lincomycin; Oxa, oxacillin; Meth, methicillin; Pen G, penicillin G; Clinda, clindamycin; Naf, nafcillin.

Except for the N variants, all of the antibiotic susceptibility results were identical. The zone sizes of the SCV were slightly larger than the parental strains in a majority (60%) of the cases. Whether this represents slight differences in response to the antibiotics or results from the fact that antibiotics on the SCV plates diffuse greater distances before the slow-growing SCV begin to develop is not known.

Hugo and Stretton (9) demonstrated that antibiotic resistance increased in direct proportion to an increase in cellular lipids of *S. aureus*. Therefore, a correlation was established between antibiotic resistance and the ETS via the cellular lipids. This type of relationship might occur in vivo and lead to changes in sensitivities of gram-positive organisms.

The formation of antibiotic-resistant SCV could, of course, lead to persistent infections that would not respond well to treatment. Of even greater significance is the fact that the clinical microbiologist could easily overlook de-

tecting SCV upon primary isolation since they are extremely small on routine culture media. A recent history of recurrent infection or difficulty in isolation of an agent should alert the microbiologist to the possibility that SCV may exist in vivo. Any minute colonies observed on the routine media, in such suspect cases, should be tested with the screening procedure described in this study, using the most common growth-stimulating factors. Successful stimulation of the minute colonies by one or more of the factors confirms the existence of SCV and would allow new susceptibility patterns to be determined.

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