Growth of *Staphylococcus epidermidis* in Soft Agar in Relation to Respiration, Dehydrogenase Activity and Biotype

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Using 200 fresh isolates of *Staphylococcus epidermidis*, the relationship between type of growth in soft-agar medium and respiration, dehydrogenase activity and biotype was investigated. When strains of *S. epidermidis* were cultured in Brain Heart Infusion medium containing 0.15% (w/v) agar, the following different growth types were observed: compact colonial morphology with growth throughout the medium (type A), or with growth only at the surface (type B); and diffuse colonial morphology with growth throughout the medium (type C), growth only at the surface (type D), or growth from the surface to the middle of the tube (type E). Five representative strains of each growth type were studied and different results for cytochrome pattern, oxygen consumption and relative activities of lactic dehydrogenase and succinic dehydrogenase were obtained with different growth types. However, there was no correlation between growth type and biotype.

INTRODUCTION

Presence or absence of oxygen is regarded as an important determining factor in the metabolism of bacteria, especially *Pasteurella pestis* (Englesberg *et al.*, 1954), *Micrococcus* (Kocur & Mortensen, 1967), *Streptococcus pneumoniae* (Howden, 1976), *Staphylococcus aureus* (Frerman & White, 1967; Goldenbaum & White, 1974), and *Staphylococcus epidermidis* (Jacobs *et al.*, 1963, Horan *et al.*, 1978). A change of oxygen requirements in multiplying organisms is assumed to reflect alteration in the cytochrome type (Schaeffer, 1952; Jacobs & Conti, 1965) and respiration capacity (Moss, 1956; Collins & Lascelles, 1962).

Finkelstein & Sulkin (1958) described a method for differentiating colonial morphologies of staphylococci under facultative anaerobic conditions in serum-soft agar (SSA). Yoshida *et al.* (1971) observed compact-type growth of *S. epidermidis* strains in SSA although diffuse-type growth was regarded as the typical colonial morphology for this species. This technique was later applied to taxonomic studies (Evans & Kloos, 1972), serological typing (Yoshida *et al.*, 1972; Nishine *et al.*, 1976), and to the investigation of cell surface properties (Takahashi *et al.*, 1977; Yoshida *et al.*, 1977; Ohtomo *et al.*, 1981). Recently we observed a variety of growth conditions and colonial morphologies of *S. epidermidis* strains in plain soft-agar (SA) medium. Attempts were therefore made to determine whether these findings relate to respiration systems and biotype.

METHODS

Strains. The 200 strains of *S. epidermidis* used were freshly isolated from clinical specimens at the Bacteriology Section, Clinical Laboratory, St Marianna University Medical School Hospital, Kawasaki, Japan. All strains were positive for catalase and arginine dehydrolase, all oxidized and fermented glucose as determined by OF medium (Difco). They were negative for tube coagulase, clumping factor reaction and deoxyribonuclease, and all strains oxidized and fermented trehalose. Except for the strains that belonged to biotypes 3 and 4 (*sensu* Baird-

Fig. 1. Colonial morphologies and growth pattern of strains of *S. aureus* and *S. epidermidis* in soft agar. The designations *S. aureus*, A, B, C, D and E refer to the compact variant of *S. aureus* and representative strains of growth types A, B, C, D and E of *S. epidermidis*, respectively.

Parker, 1974), they were positive for phosphatase by the method of Pennock & Huddy (1967) and sensitive to novobiocin; the strains gave variable results when tested for nitrate reduction by the methods of Oeding & Digranes (1977) and Kloos & Schleifer (1975). On the basis of these results, they were identified as *S. epidermidis* (*sensu* Kloos & Schleifer, 1975). The compact variant of *S. aureus* strain Smith (Yoshida *et al.*, 1974) was used as control.

Soft-agar technique. The SA technique was used as described previously (Yoshida, 1973). Organisms, grown in Brain Heart Infusion broth (BHI, Difco) at 37 °C overnight, were diluted $1:10^6$ with sterile saline; 0.1 ml of this cell suspension was combined with 10 ml BHI (pH 7.4) containing 0.15% (w/v) agar (Bacto-agar, Difco) and incubated at 37 °C for 24 h, after which colonial morphologies were determined. In these experiments strains exhibiting compact colonial morphology and growing throughout the medium were designated growth type A and those growing only near the surface were designated growth type B. Strains showing a diffuse type of colonial morphology and growing throughout the medium were designated growth type C; diffuse colonies growing only near the surface were designated growth type D, while those growing from the surface to the middle of the tube were designated growth type E (Fig. 1). Growth type of each strain was checked three times and found to be reproducible. Five randomly selected strains from each type were examined for cytochrome content, oxygen consumption and enzyme activity.

Preparation of whole cell extract. Strains inoculated into 500 ml BHI broth (pH 7.4) were cultured at 37 °C for 24 h. After harvesting by centrifugation (7000 *g*) at 4 °C, cell pellets were mixed with 10 ml glass beads (0.17 mm, B. Braun Apparatebau, Melsungen, F.R.G.) and 20 ml 0.3 M-phosphate buffer (pH 7.4) and then disrupted using a Braun cell disintegrator (type 853031) at 3000 rev. min⁻¹ for 15 min. The slurry was filtered through a sintered glass filter and centrifuged at 10000 *g* at 4 °C for 30 min. Supernatants contained approximately 3 mg protein ml⁻¹ determined by the Lowry method and were stored at -30 °C.

Identification of cytochrome types. Cytochrome types were identified by the methods of Jacobs & Conti (1965) and Frerman & White (1967) using a spectrophotometer (type LIV-300, Shimadzu, Co., Kyoto, Japan).

Assay for oxygen consumption. Oxygen consumption (Q_{O_2}) was measured by a Warburg technique at 37 °C. Each Warburg vessel contained 0.1 ml 20% (w/v) KOH in the centre well. The Q_{O_2} value was expressed as $\mu\text{l O}_2 \text{ h}^{-1} (\text{mg dry wt})^{-1}$ and was calculated from the linear portion of the oxygen uptake curve.

Enzyme assays. Relative lactic dehydrogenase activity was measured in a spectrophotometer (model UV-200, Shimadzu Co., Kyoto, Japan) by the method of Allen (1961). The reaction mixture contained 1.0 ml sodium lactate

(saturated solution), 22 ml 0.05 M-Tris/HCl buffer (pH 7.5), 2.0 ml 0.06 M-KCN, 1 mg phenazine methylsulphate, 5 mg NAD and 4 mg *p*-nitro blue tetrazolium. Whole cell extract (0.2 ml) was added to a cuvette containing 30 ml of the solution (kept at 37 °C) and mixed rapidly. The reduction rate of the nitro blue tetrazolium was measured at 60 s intervals at 625 nm. Relative activity of succinic dehydrogenase was measured as follows by a method modified from Slater (1949). Methylene blue (1.0 ml), KCN (1.0 ml) and sodium succinate (0.8 ml) were added to the reaction mixture in a 20 ml Thunberg tube containing 9.0 ml 0.1 M-phosphate buffer (pH 7.5). Diluted whole-cell extract was placed in the side-arm and activity was determined by the reduction rate of methylene blue at 380 nm using a spectrophotometer (Spectronic-20, Shimadzu, Co., Kyoto, Japan). Distilled water without cell extract was used in control tubes.

Examination of the biotype. Strains were biotyped by the method of Baird-Parker (1974). Production of acetoin was determined by the procedure of Baird-Parker (1974) and aerobic acid production from lactose, maltose, and mannitol was examined by the method of Hugh & Leifson (1953).

RESULTS

Growth type of S. epidermidis in soft agar

Two hundred strains of *S. epidermidis* were assigned to growth types as follows: type A (21), type B (8), type C (66), type D (33) and type E (72) (Table 1). Growth types A and C, which grow throughout the medium, would be expected to be facultatively anaerobic, while the behaviour of types B, D and E, in which colonies were restricted to the top of the medium, is presumed to reflect a degree of O₂ dependence (Fig. 1).

Relation of growth type to cytochrome

Cell-free extracts obtained from five representative strains of each growth type exhibited generally similar absorption spectra between 450 and 650 nm. However, differences were noted at the following wavelengths: 440, 493, 524, 557, 558, 573, 600 and 602 nm (Fig. 2). It is notable that the spectra of cell-free extracts from growth type A and C strains showed similar degrees of absorption at 440, 524, 558 and 600 nm. However an additional minor absorption peak at 510 nm was detected with growth type C. The absorption maxima of strains of other growth types were: type B, 440, 473, 524, 558, and 602 nm; type D, 440, 528, 557, 573 and 602 nm; type E, 440, 528, 557, 588 and 602 nm. The compact variant of *S. aureus* Smith strain revealed different spectra from those of *S. epidermidis* strains. Each growth type showed characteristic spectra at 524 and/or 557 to 558 nm indicating the presence of type *a* and/or type *b* cytochromes. In strains of growth type D and E, peaks were found at 573 and 588 nm suggesting the presence of type *c* and *a*, cytochromes, respectively. However, the small peak at 493 nm observed with growth type B strains was difficult to interpret (Fig. 2).

Relation of growth type to oxygen consumption

These experiments were designed to compare the relation between growth type of *S. epidermidis* strains in SA and respiration capacities. Oxygen consumption rates (expressed as Q_{O_2} ; μ l O₂

Table 1. *Relation of growth type in soft-agar to biotype of strains of S. epidermidis*

Biotype	No. of strains of growth type*					Total	Biotype as percentage of total
	A	B	C	D	E		
1	0	0	17	0	45	62	31.0
2	0	0	0	0	1	1	0.5
3	0	0	2	6	0	8	4.0
4	0	8	7	14	4	33	16.5
Non-typable	21	0	40	13	22	96	48.0
Total	21	8	66	33	72	200	100.0
Growth type as percentage of total	10.5	4.0	33.0	16.5	36.0	100.0	

* The growth type of each strain was checked on three occasions.

Fig. 2. Examples of absorption spectra of a strain of a compact variant of *S. aureus* strain Smith and five different growth types of *S. epidermidis* in soft agar (A-E).

Fig. 3

Fig. 3. Succinic dehydrogenase activities of whole-cell extracts of the compact variant of Smith strain of *S. aureus* (○) and representative strains of *S. epidermidis* of growth types A (●), B (△), C (▲), D (□) and E (■) in soft agar; control, no cell extract added (▼). Values represent mean $\pm$ S.E. of four determinations.

Fig. 4

Fig. 4. Lactic dehydrogenase activities of whole-cell extracts of the compact variant of Smith strain of *S. aureus* (○) and representative strains of *S. epidermidis* of growth types A (●), B (△), C (▲), D (□) and E (■) in soft agar; control, no cell extract added (▼). Values represent mean $\pm$ S.E. of four determinations.

$\text{h}^{-1} (\text{mg dry wt})^{-1}$ increased as follows (mean values $\pm$ S.D.): 79 ± 3.1 , 66 ± 2.8 , 59 ± 4.2 , 52 ± 1.5 , 49 ± 2.9 and $38 \pm 3.8 Q_{O_2}$, for preparations from strains of *S. aureus* and of *S. epidermidis* growth types A, C, E, D and B, respectively. These results agreed with macroscopic findings of aerobic and anaerobic growth of the organisms in SA medium.

Relation of growth type to dehydrogenase activity

A similar pattern of results was observed among the same growth type strains for relative activity of lactic dehydrogenase and succinic dehydrogenase. However, growth types A and C (facultatively anaerobic) showed higher activities than B, D and E (aerobic) (Figs 3 and 4). The compact variant of Smith strain of *S. aureus* showed considerably higher lactic dehydrogenase and succinic dehydrogenase activity than any of the *S. epidermidis* growth types. These results agreed with the results obtained for oxygen consumption described above.

Relation of growth type to biotype

None of 21 strains of growth type A could be classified according to biotype and all 8 strains of growth type B belonged to biotype 4. Of 66 strains of growth type C, 17 (26%), 2 (3%) and 7 strains (11%) were of biotype 1, 3 and 4, respectively, and 40 strains (61%) were non-typable. Of 33 strains of growth type D, 6 (18%), 14 (42%) and 13 (40%) strains were of biotype 3, 4 and non-typable, respectively. Of the 72 strains of growth type E, 45 (63%), 1 (1%), 4 (6%) and 22 (31%) strains were of biotype 1, 2, 3 and non-typable, respectively, indicating that there was no significant correlation between growth type and biotype.

DISCUSSION

Variation in biosynthesis and composition of the membrane-bound respiratory chain in growing staphylococci was related to oxygen tension (Jacobs *et al.*, 1967; Frerman & White, 1967). Also, greater cytochrome content and nitrate reducing ability in aerobically grown strains of *S. epidermidis* compared with anaerobically grown cultures has been reported (Jacobs *et al.*, 1963; Jacobs & Conti, 1965). In experiments reported here, cytochrome patterns of *S. epidermidis* strains belonging to growth types A and C, which were facultatively anaerobic, agreed with reports for *S. epidermidis* by Jacobs & Conti (1965) and for *S. aureus* (Goldenbaum & White, 1974). The specific absorbances at 493, 573 and 588 nm of strains of growth type B, D and E, which were aerobic, differed from each other and from types A and C. In addition, the oxygen requirements of the various growth types of *S. epidermidis* strains correlated with the cytochrome patterns, suggesting a possible correlation between cytochrome systems and respiration capacity as postulated by Jacobs & Conti (1965).

Gotz & Schleifer (1978) and Sivakanesan & Dawes (1980) noted higher dehydrogenase activities during aerobic growth of an *S. epidermidis* strain than during anaerobic growth. Also, Collins & Lascelles (1962) and Stockland & San Clemente (1968) found that dehydrogenase activity of aerobically cultured strains of *S. aureus* was higher than that of anaerobically grown cells. Relative activities of lactic dehydrogenase and succinate dehydrogenase obtained in our experiments were higher in strains that showed higher oxygen consumption. However, from these experiments it appeared that aerobic strains do not necessarily show greater dehydrogenase production and oxygen consumption than strains of the facultatively anaerobic growth type.

Finkelstein & Sulkin (1958) reported that diffuse-type growth is a specific type of colonial morphology of *S. epidermidis* in SSA. However, Yoshida *et al.* (1971) noted compact-type strains of *S. epidermidis* in this medium and later Usui *et al.* (1979) obtained a specific substance from these organisms which reacted with serum components. Forsum *et al.* (1972) reported on the existence of compact-type strains in plain SA, whilst Evans & Kloos (1972) showed various types of growth of members of the genera *Micrococcus* and *Staphylococcus* in an anaerobic zone of a SA medium. In our experiments five different growth types of the strains were observed in *S. epidermidis*, which were similar to those of strains of group A streptococci (Whittenbury, 1978). The mechanism of compact-colony formation of *S. aureus* and some strains of *S. epidermidis* in SSA has already been elucidated by Yoshida *et al.* (1971) and Usui *et al.* (1979). In contrast, the mechanism of the appearance of different colonial morphologies of *S. epidermidis* in plain SA is unknown; however, there was no significant relation between colonial morphologies and biotype. The biological significance of growth type of the strains and possible classification of other species within the genus *Staphylococcus* require to be elucidated; such studies are currently being performed in our laboratory.

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