Scanning Electron Microscopy of Intact Colonies of Microorganisms

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Colonies of *S. mutans* OMZ61, *Streptococcus* sp. D182, *Staphylococcus aureus* Oxford NCTC 6571, and *Candida albicans* type A, MRL 3153 were grown on various media. Cubes of agar bearing two to three colonies were excised and processed for scanning electron microscopy. The characteristic shape of the colonies was seen when examined at low magnifications. At a magnification of 2,000 diameters, the arrangement of individual organisms within the colonies was observed. Plano-convex colonies consisted of uniformly distributed organisms, whereas *S. mutans* colonies presented a more complex arrangement possibly associated with the production of extracellular polysaccharides. Certain colonies were totally or partially covered by an adherent film through which the outline of the organisms could be distinguished.

The colonial morphology of bacteria has been used as an aid to the identification of bacteria (Berger's *Manual*, 7th ed., p. 508–509) since the time of Koch. Little, however, appears to be known about the relationship between the morphology of the whole colony and its microstructure, although, recently, it has been possible to derive mathematical expressions for the growth of various organisms, including *Escherichia coli*, *Klebsiella aerogenes* and *Streptococcus faecalis* on agar media (5, 17).

Colonial morphology is a function of many parameters. For example, *S. pneumoniae* is able to produce either smooth or rough colonies, depending on whether the constituent cells are capsulated (21), whereas *S. mutans* OMZ61 produces a plano-convex colony on blood-agar but a raised, irregular colony on mitis salivarius agar (6). Conventional light microscopy yields little information about the internal arrangement of colonies of smaller microorganisms, since there is insufficient depth of field at higher magnifications to enable spatial relationships between the latter to be determined, although light microscopy has been used to study colony growth of the considerably larger microorganism, *Chaetomium* (18).

To gain further information on individual organisms, sections through araldite-embedded colonies have recently been examined by using transmission electron microscopy (12, 20), although this approach fails to provide data on the three-dimensional structure of colonies.

The scanning electron microscope, however, appears to be ideally suited to this kind of study since resolution is approximately 250 nm and depth of field is approximately 200X that of the light microscope (2, 4). The usefulness of this instrument in biology has been largely restricted to studies of mineralized and relatively rigid tissues (13), although more recently techniques suitable for soft tissue preparations have become available (7). Williams and Davies (22) used a scanning electron microscope in the examination of *Actinomyces* but were not concerned with either whole colonies or with colony structure. More recently, Klainer and Betsch (11) examined bacteria, grown in liquid media, by scanning electron microscope, and the instrument was used to study bacterial spores (3, 16). Bacterial colonies and the solid media on which they grow are delicate structures easily distorted during preparation for the scanning electron microscope.

In the present study, a critical examination is made of several techniques for preparation of colonies for the scanning electron microscope. Results on four types of microorganisms grown on various media are presented.

**MATERIALS AND METHODS**

Organisms and cultivation. The organisms examined were the following: *S. mutans* OMZ61 obtained from B. Guggenheim, Zurich; *Streptococcus* sp. D182, a noncariogenic *Streptococcus* isolated in this laboratory; *Staphylococcus aureus* Oxford NCTC
6571 obtained from the National Collection Type Cultures, Colindale; Candida albicans type A, MRL 3153 obtained from the London School of Tropical Medicine.

S. mutans and Streptococcus sp. D182 were grown on Sabouraud's agar (Oxoid). All test organisms were incubated for 48 hr on agar plates of standard thickness (0.4 cm).

Fixation and preparation of colonies. Cubes of agar approximately 0.5 by 0.3 cm and bearing two to three colonies were excised from the plates and measured in two dimensions by a micrometer eyepiece. Selected colonies on the blocks were measured across two diameters at right angles to each other and photographed at a standard magnification of 40X. Colonies of the various organisms were divided into four groups and subjected to one of the following techniques.

Group I colonies were fixed for 2 hr in 3% glutaraldehyde in phosphate buffer (10) at 2°C and washed in four changes of distilled water, the last change being overnight. They were quenched in isopentane-cooled in liquid nitrogen and freeze-dried for 24 hr at −40°C at 10⁻³ Torr or 6 hr at −40°C at 10⁻⁸ Torr.

Group II colonies were treated in the same manner except that the quenching in isopentane was omitted and specimens were transferred directly to the freeze drier from distilled water.

Group III colonies were not fixed and washed but were quenched in isopentane in liquid nitrogen immediately after measurement and photography.

Group IV colonies were fixed and washed but dehydrated in ascending concentrations of alcohol instead of by freeze drying. The blocks remained at concentrations of alcohol of 70, 80, 90 and 100% for 15 min and were finally air-dried.

Scanning electron microscopy. The dried agar cubes were cemented on to aluminum stubs and coated with a gold palladium (60–40) alloy evaporated on to their surfaces while rotating in a vacuum of 10⁻⁶ Torr. The agar cubes and colonies were remeasured and photographed, and the percentage of linear distortion in two dimensions was calculated. The colonies were examined in a Cambridge Instrument Stereoscan at 40 kv and various magnifications.

RESULTS

Specimens which were alcohol-dehydrated (group IV) and those freeze-dried without quenching (group II) were severely distorted; these methods, therefore, were not pursued.

Shrinkage of the agar blocks occurred to some extent in all the methods examined.

The average linear shrinkage of group I agar cubes was 14.8%, whereas that for group III cubes was 23.1%. In the case of the colonies themselves, prior fixation followed by quenching and freeze-drying (group I) produced an average decrease in diameter of 23%, whereas quenching alone followed by freeze-drying (group III) produced a decrease in diameter of 11.2%. Some of the colonies processed in group III showed no distortion, either by micrometer eyepiece estimation or by comparison of the pre- and post-treatment photographs (Fig. 1).

Colonies processed by the group I and III methods were dimensionally and morphologically stable.

Certain colonies, however (particularly C. albicans), were disrupted by fixation and washing and were therefore only prepared by the group III method. This method produced least shrinkage and morphological change and was applicable to all the colonies studied.

At low magnifications, the characteristic morphology of the colonies was maintained (Fig. 2).

Examination at high magnification revealed variations in arrangement of the organisms in different colony types and in different areas of the same colony.

Organisms in the central depressed area of the S. mutans colonies were arranged in chains grouped together to form broad intercommunicating buttresses with spaces between producing a spongelike effect (Fig. 3). In several colonies, the organisms were coated with a gummy material which appeared to cement them together and which obliterated their outlines in some areas. In the raised periphery of these colonies, the organisms which were on average 1.4 by 0.8 μm were much more closely packed, and no cementing or coating material was visible. Incomplete division of two organisms was often apparent when the total length was up to 2.2 μm. The organisms had a banded appearance; the periodicity of the banding was approximately 0.2 μm (Fig. 4).

The domed colonies of Streptococcus sp. D182 were composed of tightly arranged, almost spherical organisms approximately 0.75 μm in diameter. Bridging the gap between adjacent bacteria, fine filamentous strands could be seen which may have been extensions of the cell wall material (Fig. 5).

The arrangement of bacteria in the domed S. aureus colonies was exactly similar to the Streptococcus sp. D182 colonies. The organisms were approximately 0.75 μm in diameter, spherical in shape, and tightly packed. Covering part of the colony surface was a film through which the faint outlines of the organisms could be distinguished.

The domed colonies of C. albicans invariably became detached from their supporting agar but could still, with care, be mounted for examination in the scanning electron microscope.

The entire surface of these colonies was covered by a thin film less than 0.1 μm in thickness through which the outlines of the spherical organisms
could be seen. One or more small drying cracks enabled the interior of the colony to be seen. The interior was composed of packed spherical organisms approximately 2.5 μm in diameter. Fine filamentous strands associated with the surfaces of the organisms were evident, and these were more numerous immediately below the film covering the colony surface (Fig. 6).

One C. albicans colony had become inverted during processing and an examination of the under surface revealed organisms much less uniform in size and shape than those nearer the surface. Diameters ranged from 1 to 4 μm, and organisms displaying budding were numerous (Fig. 7).

**DISCUSSION**

Quenching in isopentane in liquid nitrogen without prior fixation, followed by freeze drying, offers a satisfactory method of preparation of colonies for scanning electron microscopy. Millington et al. (15) used a similar technique for their study of intestinal mucosa; most previous workers, however, favored chemical fixation usually followed by freeze-drying (8, 14, 19) or acetone dehydration (1).

Our results indicate that for friable bacterial colonies dehydration by freeze-drying was satisfactory. Although prior fixation in glutaraldehyde or direct quenching of nonfixed material were suitable for some colonies, the majority were disrupted by chemical fixatives. Direct quenching of nonfixed material was applicable to all the colony types studied and presently appears to be the method of choice for this particular biological material.

Zachariah et al. (24) recently proposed a much simpler technique of freeze-drying than the one used in this study; their protozoal material is probably more robust, however, than either the bacterial colonies themselves or the agar on which they are growing.

**FIG. 1.** *Streptococcus mutans* OMZ61 colony. (A) Colony growing on mitis-salivarius agar. (B) Same colony after drying and metallic coating in preparation for scanning electron microscopy. Note the dimensional and morphological stability. Photographed by conventional light microscopy. Bar represents 300 μm.
Fig. 2. *Streptococcus* sp. D182 examined in the scanning electron microscope. The preparation technique has preserved the plano-convex character of the colony. Bar represents 100 µm.

The results of our high-power studies in the scanning electron microscope appear to indicate that a relationship exists between colonial morphology and internal arrangement of bacteria within a colony, since the plano-convex colonies of *S. aureus*, *C. albicans*, *Streptococcus* sp. D182 typically consisted of uniformly arranged microorganisms; the more complex colonies of *Streptococcus mutans*, however, possessed more complex internal arrangements.

The open arrangement of the central area of *S. mutans* colonies may be due to the production of extracellular glycans from sucrose present in the medium (23). These glycans could conceivably prevent the bacteria from growing too closely together, in which case the drying process would account for the spongeliike appearance of the colonies, spaces being produced upon desiccation of the extracellular glycans. The raised rim of *S. mutans* colonies, consisting of tightly packed organisms, forms at a later stage in colony development when conditions may be less favorable for glycan production. The nonspherical shape of individual cells is not surprising in view of the work of Swanson and McCarty (20), and the banded appearance may prove, in the light of recent findings (9), to be due to sequential synthesis of cell wall material before septum formation, although the separation of bands is much less than one would expect from theoretical considerations. An illustration of such banding appears in a recent paper (11), although the banding is not described within the text.

The thin strands attached to cells of *Streptococcus* sp. D182 may be either (i) the result of drying of extracellular materials or (ii) intercellular bridges of the type reported by Klainer and Betsch (11). The simplicity of the arrangement of the bacteria is reflected in the appearance
FIG. 3. Central depressed area of a Streptococcus mutans OMZ61 colony examined in the scanning electron microscope. Note the spongelike arrangement of the organisms. Compare with Fig. 4. Bar represents 5 μm.

FIG. 4. Scanning electron micrograph of organisms in the raised periphery of a Streptococcus mutans OMZ61 colony. Note the banded appearance and tightly packed arrangement of the bacteria. Bar represents 5 μm.
FIG. 5. Domed Streptococcus sp. D182 colonies are made of tightly arranged, almost spherical organisms. Fine filamentous strands between the bacteria can be seen on this scanning electron micrograph. Bar represents 5 μm.

FIG. 6. C. albicans colony examined in the scanning electron microscope. High-power view of shrinkage crack. Note the surface covering film and the tightly arranged spherical organisms bearing filamentous strands. Bar represents 10 μm.
of the colony, which is probably the simplest possible colony form. The same considerations apply to colonies of *S. aureus*, although, in this case, a covering film was shown to be present. The film probably arises during life from the drying out of materials at the colony's surface. It is probably not a preparation artefact, however, since films were typically associated with young colonies and were frequently ruptured on older colonies.

*C. albicans* colonies invariably refused to adhere to the agar on drying, probably because this organism fails either to grow into the agar medium or to produce adhesive extracellular material of the type produced by *S. mutans*. The film covering *C. albicans* colonies presumably arose in much the same way as that of *S. aureus*, whereas the filamentous strands attached to individual cells may have arisen in much the same way as those found in *Streptococcus* sp. D182 colonies or they may be “budding scars.”

The finding that cells of *C. albicans* appear to be budding only at the base of the colony suggests that growth occurs mainly at the base of the colony, at which place nutrient is most plentiful, rather than evenly throughout the colony in accordance with the laws governing the growth of microorganisms in liquid culture.

Calculations on colonial growth were based on two assumptions: (i) that even growth occurs throughout colonies and (ii) that organisms are evenly distributed within colonies. However, our work reveals that, in the case of *C. albicans*, even growth does not take place throughout colonies and, in the case of *Streptococcus mutans*, the organisms are not evenly distributed within colonies.

Further work is in progress to determine how colonial morphology varies with time and to elucidate the factors governing such change in morphology.

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**LITERATURE CITED**


