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# Culture Purity Assessments and Morphological Dissociation in the Pleomorphic Microorganism *Bacterionema matruchotii*

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The inherent pleomorphism of *Bacterionema matruchotii* resulting from its mode of reproduction was enhanced by temporal effects of culture age and growth condition and by the more lasting effects of rough to intermediate to smooth morphological dissociation. Routine morphological observations with a single growth condition were inadequate to permit unambiguous judgements of culture purity. Multiple criteria were required. Pleomorphism and the undetected presence of contaminants in primary and successive cultures of *B. matruchotii* can explain the emergence of unrelated bacteria from *B. matruchotii* reported previously by others and ascribed to genetic instability.

Morphological variability exhibited by *Bacterionema matruchotii*, a filamentous microorganism indigenous to the oral flora, is a consequence of its mode of reproduction (9), cultural environment (10), and an apparent rough to intermediate to smooth (R → I → S) morphological dissociation (2). This pleomorphism should reduce the accuracy of culture purity assessments which depend upon routine observations of colonial and cell morphologies in a restricted number of cultural conditions.

Recent reports describe the emergence of genetically different bacillary and coccal "variants" from *B. matruchotii* (7, 19, 20). Gram-negative rod elements released during serial broth transfer were associated with the development of diphtheroid "variants" which formed flat small colonies on plates (20; J. L. Streckfuss, W. N. Smith, A. C. Taylor, and J. Ennever, Int. Ass. Dent. Res. Abstr., no. 192, 1971). The diphtheroid "variants" were considered to be a genetically unstable intermediate phase because those which were subculturable yielded different and stable variants, some of which were identified as *Streptococcus* sp. (*mutans*, *sanguis*, and *mitis*) and *Lactobacillus* sp. (W. N. Smith, J. L. Streckfuss, and L. R. Brown, Int. Ass. Dent. Res. Abstr., no. 59, 1971). "Variant" formation was proposed to occur as a result of genetic instability in *B. matruchotii* rather than from contamination or the interaction between species and was termed "morphological dissociation" (20).

This interpretation of unique genetic instability in *B. matruchotii* implies that extreme variability should occur both between and within pure culture isolates. If this were true, pure culture assessments and correlative genetic factors and taxonomic concepts would be highly complicated. However, with more than 200 aerobically derived and tested strains isolated in the U.S.A., England, and Japan, there was a remarkable degree of homogeneity and reproducibility in some 28 simple biochemical tests, and only one serotype has been reported (M. N. Gilmour, *In* R. Buchanan and N. Gibbons, ed., *Bergey's Manual of Determinative Bacteriology*, 8th ed., in press). Also, the development of "variants" as described by Streckfuss and Smith (20) has not been seen during observations of the growth of more than 500 single cells to microcolonies (9; M. N. Gilmour and G. Turner, unpublished data).

These discrepancies instigated the current studies. (i) The effects of cultivation conditions and R → S transitions on morphology and other characteristics of *B. matruchotii* were reexamined. (ii) The emergence of coccal and bacillary "variants" was studied using the exact experimental conditions of Streckfuss and Smith (20), since culture instability usually correlates with culture history and handling (3). (iii) The colony-forming unit (CFU) recoveries of *B. matruchotii* and a diphtheroid similar to the above-described intermediate diphtheroid "variant" were compared in pure and in mixed cultures. (iv) Culture purity assessments based on conventional observations were shown to be inadequate, and the need for nonroutine procedure is discussed.

## MATERIALS AND METHODS

**Cultures and maintenance.** Table 1 lists cultures, which were kindly provided by J. L. Streckfuss, and

their lineage and history with respect to "variant" production. Original isolation of the two ATCC cultures was by Gilmour (10), and that of strain 13 was by Richardson and Schmidt (18). The cultures received were tested for purity immediately upon opening and again 3 days later. Additional isolates and strains utilized were obtained from dental calculus or plaque and were similar to previous descriptions of them (9–12). Working stock cultures were maintained on a half-strength brain heart infusion agar (HBHI) (10) by incubating 2 days in air supplemented with 5% $CO_2$, storing at 3 C, and transferring at 2-week intervals. For long-term storage, suspensions of early stationary-phase cultures in HBHI supplemented with 10% glycerol were heat-sealed in glass vials and stored at −75 C.

**Culture purification and purity testing.** Unless otherwise stated, purification was accomplished by: (i) serially streaking microcolonies (30 to 50 cells) three times using HBHI with 20-h carbon dioxide-supplemented aerobic incubation and/or HBHI-hemin with 30-h anaerobic incubation, followed by (ii) cultivation of approximately 40 tubes, each containing 1 ml of a broth suspension homogenized lightly to disperse the cells and diluted to contain less than 0.5 CFU/ml, (iii) recovery from cultures with 1 granule or the least amount of growth for a second or third cycle of streaking and dilution as above, and (iv) a final round of serial streaking as in (i).

Cultures were routinely tested for purity by microscopic examination of colonial and cellular morphologies of simultaneously prepared multiple cultures. To enhance the sensitivity of contaminant detection, three media were employed, and each was incubated in three gaseous environments. The media used were: HBHI supplemented with glucose to 0.5% and, for anaerobic incubation, addition of 0.05 mg of hemin per ml (13); modified tryptone with 0.5% glucose (13); and brain heart infusion (BHI, Difco). The gaseous incubation environments were 95% $H_2$ plus 5% $CO_2$ in Brewer jars, air, and air supplemented with 5% $CO_2$. Appropriate variations of these conditions were employed to subculture colonies other than *B. matruchotii* which were encountered.

Colonial morphologies were assessed microscopically both for typical mature colonies, which can vary in appearance (2, 11, 12), and for immature spider-microcolonies (12), which were found herein and elsewhere (M. N. Gilmour, unpublished data) to be a universal characteristic of all known strains of *B. matruchotii*. Cell morphologies were examined at ×1,000 (numerical aperture [N.A.] 1.35) utilizing (i) plates with microcolonies which were covered with cleaned cover slips, and (ii) smears prepared with filtered stains.

Final purity assessments included observations of growth in microcultures (9) and biochemical testing (10). The microcultures were modified by using a medium prepared by mixing equal volumes of separately sterilized double-strength HBHI broth and 4% Ionagar no. 2 (Colab Laboratories, Inc., Glenwood, Ill.) in water (pH 7.4) and adding glucose to 0.5%. Growth from single cells was observed with phase-contrast microscopy (×900, N.A. 1.35).

**"Morphological dissociation."** Experiments were performed as described by Streckfuss and Smith (20) with pure cultures of strains 1-$F_7$ $P_6$ and 15-65 $P_2$. Freshly prepared BHI broth or agar was used, and all cultures and controls were incubated in air at 37 C. Multiple CFU of 1-$F_7$ $P_6$ were used to initiate serial transfer of exponential-phase cultures of 1-$F_7$ $P_6$. To initiate cultures from a single CFU (20), spider-microcolonies from 20-h plates of strain 15-65 $P_2$ were suspended in broth, vigorously shaken,

TABLE 1. *Culture derivation and morphological dissociation history*[a]

| Culture derivation[b] | | | Culture designation | History of flat colony variant production[c] |
|---|---|---|---|---|
| ATCC 14265 → (M.N.G., no. 18) | CFU 1 (1-65) → | CFU 11 | 11-65 $P_2$[d] | $P_1$[d] ↛ variants |
| | | CFU 15 | 15-65 $P_2$ | $P_1$ → variants (1-65 ↛ variants) |
| ATCC 14266 (M.N.G., no. 47) → | CFU 17 | | 17-66 $P_2$ | $P_1$ and $P_2$ ↛ variants |
| | CFU 19 | | 19-66 $P_2$ | $P_1$ and $P_2$ ↛ variants |
| Richardson's → strain 13 | CFU 7 (by dilution) → | CFU 1 | 1-$F_7$ $P_6$ | $P_6$ ↛ variants, but $P_{10}$ → variants |
| | | CFU 2 | 2-$F_7$ $P_6$ | $P_1$-$P_{10}$ ↛ variants |

[a] Data from J. L. Streckfuss and W. N. Smith (personal communication).

[b] CFU refers to the designation of the CFU isolated either by micromanipulation or dilution.

[c] The occurrence of variant colonies was low among the cultures tested, different between strains and between CFU isolates from the same strain, and possibly independent of the number of serial broth passages, because variants were found as early as the first broth passage of the CFU 15-65 but not until the tenth with 1-$F_7$.

[d] P, serial exponential-phase broth passages numbered consecutively.

and diluted, and multiple flasks were each inoculated with approximately 1 CFU which was ascertained from viable counts and the incidence of granules in the broth cultures. The resulting culture was used to initiate serial broth-to-broth passage of exponential-phase cultures. To reduce the possibility of contaminant entry (3), a duplicate culture was made at each passage for testing "variant" production. These cultures were incubated for 40 h and four serial dilutions from them were plated. After 5 days of incubation, all plates, dilution tubes, original cultures, and subcultures from them were observed for any colonial and cell morphologies differing from *B. matruchotii*.

**Mixed cultures.** The effect of mixed culture growth on CFU recoveries was studied with *B. matruchotii* strain 15-65 $P_2$ and a flat diphtheroid colony which was isolated from the morphological dissociation experiment broth culture 2 of $1F_7$-$P_6$ (Table 2) and which was similar to the diphtheroid variant described by Streckfuss and Smith (20). Separate cultures of each were grown in BHI broth until mid-log phase, directly counted, and inoculated into flasks of BHI broth at diphtheroid-*B. matruchotii* cell ratios of 1:1 and 1:10. Controls included pure cultures of each microorganism and uninoculated broth. All flasks were incubated in air and sampled at intervals; Gram stains and viable plate counts, using three to six dilutions for each sample, were done.

## RESULTS

**Morphological observations.** All cultures were checked for purity as described above. Among those received, strain 15-65 $P_2$ was found to contain typical *B. matruchotii* and large populations of a diphtheroid and a coccus in both smears and cultures. The latter two were eliminated by means of rigorous purification and were not encountered over a subsequent 2-year interval, except when attributable to contaminant entry ("morphological dissociation" experiments below, Table 2). Both of the cultures derived from ATCC 14266 (Table 1) appeared to contain minor diphtheroid and coccal population components which also were effectively eliminated from the typical *B. matruchotii* component.

Morphological comparisons between the strains received, when pure, and our isolates showed that the four cultures derived from Gilmour isolates 18 and 47 (11-65 $P_2$, 15-65 $P_2$, 17-66 $P_2$, and 19-66 $P_2$, Table 1) were similar to

TABLE 2. *Relationship between plating errors and occurrence of aberrant and flat diphtheroid-containing colonies in B. matruchotii cultures*

| Inoculum | | | Plating | | | Plates with aberrant colonies | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | No. of plates with | | |
| Strain | Source | Serial broth tested | Operator[b] | Face mask[b] | No. of plates | All aberrant types | Flat diphtheroids | From dilution tube no. |
| 15-65 $P_2$ | CFU 1 | 2 | B | – | 12 | 1 | 1 | 1 |
| | | 3 | B | – | 12 | 1 | 0 | 1 |
| | CFU 3 | 1 | B | – | 8 | 1 | 0 | 3 |
| | CFU 7 | 1 | B | – | 12 | 1 | 0 | 4[c] |
| | | 2 | B | – | 12 | 1 | 0 | 3 |
| | | 7 | B | – | 12 | 2[d] | 0 | 1 |
| | | 8 | B | – | 12 | 0 | 0 | |
| 1-$F_7$ $P_6$ | Culture received | 1 | B | – | 12 | 0 | 0 | |
| | after serial streaking | 1 | B | – | 9 | 0 | 0 | |
| | | 2 | B | – | 12 | 3 | 1 | 1,4 |
| | | 3 | B | – | 12 | 1 | 0 | 2 |
| | | 4 | B | – | 12 | 9[d] | 1 | 1,2,3,4 |
| – | Sterile broth | 1 | B | – | 100 | 25[d] | 10 | |
| | | 2 | B | + | 100 | 12[d] | 7 | |
| | | 3 | C | + | 100 | 4 | 1 | |

[a] The first culture of the serially transferred exponential-phase broth cultures was started with single CFU of 15-65 $P_2$ and multiple CFU of 1-$F_7$ $P_6$.

[b] Operator B was a trained technician; operator C was a highly trained microbiologist. Symbols: –, no face mask used; +, face mask used.

[c] Dilution tube was contaminated with the same contaminant as on the plate.

[d] Contaminant present on one uninoculated plate was obviously spread and carried on the spreader to other plates.

our cultures of 18 and 47 when they were serially transferred on BHI and incubated aerobically. Aerobic mature colonies on BHI were raised with a high convoluted surface resembling a molar tooth. They were white, opaque, and friable, and tended to have a lobate edge and sometimes a fine lacerate halo. The cells were shorter, some filaments had bulbous ends which eventually lysed, and branching and pleomorphic forms were more common than when cultivated on other media or under reduced oxygen tension. With anaerobic incubation on HBHI, mature colonies were tough, filamentous, lacerate, and flat to umbonate, and cellular components included many long filaments attached to or separated from wider bacillary elements. Under all conditions, very young immature colonies were spider-like. Growth in broth was granular or in loose flakes and was readily dispersed with light homogenization. The mode of reproduction, observed in microcultures, and biochemical reactions tested at three different times were as previously described (9, 10).

Cultures 1-$F_7$ $P_6$ and 2-$F_7$ $P_6$, which derived from Richardson's strain 13 (Table 1), had the same biochemical characteristics and mode of reproduction, which resulted in spider-like microcolonies, but had certain morphological differences. Broth culture growth was diffuse. Aerobic mature colonies on BHI were butyrous, low convex, and usually with an entire edge and were composed of diphtheroid-like cells, as illustrated for the $RF_7$ bacillary "variant" by Streckfuss and Smith (see Fig. 4 and 5, ref. 20). The incidence of long rods and oval bacillary elements with deeply staining large intracellular inclusions varied with incubation time, and branching and bizarre pleomorphic morphologies increased markedly with culture age. Long filaments and the typical whip-handle cells were extremely rare, which was consistent with microculture observations of aerobically maintained and incubated cultures wherein fragmentation occurred earlier than usual, thereby preventing the formation of long filaments. A change to a more typical cellular morphology occurred in very young cultures and, with serial transfer, under anaerobic conditions on HBHI where colonies eventually became more filamentous and less butyrous and comprised higher proportions of longer filaments and whip-handle cells. These differences in colonial and cellular morphologies between 1-$F_7$ or 2-$F_7$ and the other four cultures received were more stable than the temporal effects resulting from culture age and growth condition and, in fact, resembled those described between rough to intermediate and smooth colony variants described by Bibby and Berry (2).

To substantiate the occurrence of an R → I → S transition, 20 different pure culture strains which exhibited the R morphology in primary culture were simultaneously maintained aerobically and anaerobically on various media for approximately 30 transfers. The results confirmed that colony transitions, with concomitant cellular morphology changes, occurred in an R (tough, adherent, and, under anaerobic conditions, flat, filamentous, and lacerate and composed of long filaments) through intermediate (crumbly, less adherent) to S (smooth, soft, nonadherent, short cells) sequence as detailed by B. G. Bibby (Ph.D. thesis, University of Rochester, Rochester, N.Y., 1935). The R → I → S transition was dependent upon cultural conditions. R colonies were maintained with anaerobiosis, particularly in neutral or alkaline environments; I colonies were found most frequently after repeated aerobic transfers and readily reverted to the R form under anaerobiosis; and S colonies developed after prolonged aerobic incubation, particularly in acid environments or on BHI. The 1-$F_7$ and 2-$F_7$ cultures were similar to S-colony variants previously illustrated by Bibby and Berry (2).

Broth culture appearance and cellular morphologies were also affected by this R → S transition as well as by culture age and growth condition. R cultures were granular and contained longer cells than S cultures where growth was diffuse. Pleomorphism increased after log phase; it was enhanced by aerobic incubation, as illustrated by Bibby and Berry (2), and it became particularly marked in aerobic BHI cultures. Such cultures of 15-65 $P_2$, 1-F $P_6$, and 50 other strains had a preponderance of gram-negative cells with intracellular gram-positive inclusions, an increase in pleomorphic, sometimes bizarre cell forms, and considerable cellular debris including minute gram-negative rod-like and gram-positive spherical elements. There was also an associated loss of viability: decreased plate counts, turbidities, and transferability.

**"Morphological dissociation" yielding coccal and bacillary "variants."** Morphological dissociation experiments were performed with serially transferred broth cultures of strains 15-65 $P_2$ and 1-$F_7$ $P_6$ considered to be pure by the criteria given in methods above. Colonies other than *B. matruchotii* were found on many of the plates, and their incidence was independent of initiation from single or multiple

CFU (Table 2). In general, one plate from each series had one such colony on it, but some plates had many which originated from carry-over by the spreader to successive plates. There was no correlation between the sequence of the dilution tube plated and the occurrence of non-*B. matruchotii* colonies, including those similar to the diphtheroid "variants" described by Streckfuss and Smith (see Fig. 1, ref. 20). The occurrence of such colonies could be discontinuous among the broth passages, the colony type found varied between and within plates from the individual broth cultures, and there was no relationship between the frequencies with which non-*B. matruchotii* colonies occurred and either the percentage of viable count or the *B. matruchotii* densities.

In fact, the overall incidence of non-*B. matruchotii* colonies on the morphological dissociation test plates approximated that found on plates inoculated with sterile broth (operator B, no face mask, Table 2), and the types of colonies on both sets of plates were similar. The source of these contaminants was indicated to be operator-related, because the incidence of contaminated control plates was significantly reduced to 12% ($P < .05$) when operator B wore a face mask, and it was further significantly reduced to 4% ($P < .05$) when operator C was employed for plate preparation and streaking (Table 2). Moreover, comparisons of the relative proportions of small, flat, translucent diphtheroid colonies with gram-positive, coccal-containing colonies among the contaminants and in the nares populations were similar within each of the operators B and C, but were different between them.

Stains and subcultures of each of the small, flat, translucent colonies encountered indicated that the majority were diphtheroids which had colony and cellular morphologies exactly as illustrated for the unstable diphtheroidal "variant" (see Fig. 1 and 3, ref. 20). These were transferable and, when purified, did not produce variants of differing genera. The remainder were streptococci, some of which did not subculture on BHI under aerobic conditions.

**CFU recoveries from mixed cultures.** Because of these results, the plating recoveries from mixed broth cultures of a diphtheroid similar to the described morphological dissociation "variant" and *B. matruchotii* were studied. The input inoculum ratio sizes of diphtheroid-*B. matruchotii* were 1:1 and 1:10 from direct counts and approximated 1:40 and 1:260 from viable plate counts (Fig. 1). Yields of the diphtheroid in pure cultures were higher than those in the mixtures, and they were greater in

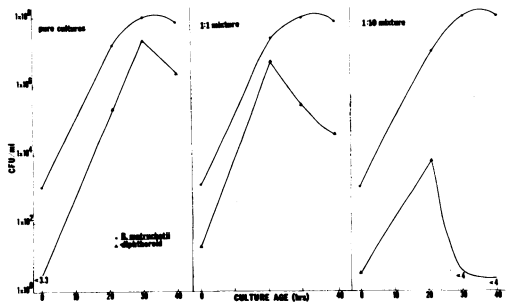

FIG. 1. *Effect of mixed culture growth with varying inocula sizes on viable counts of B. matruchotii and a diphtheroid contaminant.*

the 1:1 mixed culture than those in the 1:10, indicating growth inhibition by *B. matruchotii*. Neither the growth rate nor the CFU yields of *B. matruchotii* were affected by the diphtheroid. The relationship between the growth rate of both organisms was such that the diphtheroid could be carried by serial transfer of exponential-phase cultures, but the ability to reliably detect it with the large *B. matruchotii* inocula ratio required the use of many plates per dilution plated to minimize sampling error problems, and even this procedure eventually became unsatisfactory. Plates from the uninoculated broth controls were uncontaminated throughout.

In smears prepared at each sampling time, the diphtheroid was readily differentiated from *B. matruchotii* when each was grown singly, because the diphtheroid tended to occur in loose aggregates of four to twenty cells. However, in the mixtures at 21 h, only one aggregate was observable in 0.005 ml of the undiluted 1:1 mixture and none was found in the 1:10 culture. At this time, the diphtheroid viable counts were highest (Fig. 1), indicating that the CFU must have occurred primarily as single cells. Their appearance in pure cultures closely resembled some of the rods in the *B. matruchotii* culture at 21 h or later, and therefore their discrimination by stained smears was unreliable.

**Effect of pleomorphism on culture purity assessments.** These results showed that routine morphological examinations of growth from one cultural condition were inadequate for assessing culture purity. This was also found during purification of 188 primary isolates over a 4-year interval. Each isolate had typical *B. matruchotii* colonial morphology in primary pour plates (12). Inocula of crushed cell suspensions were serially streaked with the simultaneous use of multiple growth conditions throughout, and morphologies of colonies and of cellular

components in Gram-stained smears were monitored microscopically. Although the major cellular components in the primary colonies were attributable to *B. matruchotii*, approximately 95% had minor suspicious coccal, diphtheroidal, or actinomyces-like elements in stained smears, and >50% exhibited contaminant colonies when first transferred.

Consistent aerobic cultivation on BHI resulted in a 50% loss of cultures, 38% of which resulted from cessation of growth and 12% from overgrowth by contaminants. Curiously, these losses occurred nonrandomly with respect to transfer number from primary isolation (Table 3), and they could not be associated with known experimental variables.

Among the cultures which were not lost, 10% of the original isolates which appeared pure by colony morphology and stained smears at the consecutive fifth, sixth, and seventh aerobic transfer on BHI subsequently were found to be contaminated when subcultured under different conditions (Table 3). In each of these cases, the contaminant encountered was similar to that which had been present in the homologous primary isolate. The most frequent were diphtheroids, although *Actinomyces* sp. and a streptococcus were also involved. Their eradication was successfully accomplished by (i) inoculating with microcolonies which dispersed better than mature colonies, (ii) changing the medium to HBHI and incubating in reduced oxygen atmospheres, and (iii) utilizing cycles of streaking and dilution of broth suspensions. During purification of the *B. matruchotii*, errors were made in assessing culture purity unless multiple criteria were employed. Those cultures having the following characteristics remained pure for more than 6 years. (i) Under a variety of cultural conditions, all colonial and cellular morphologies found were typical of *B. matruchotii*. (ii) Tests of a range of biochemical characteristics were reproducible when repeated a minimum of three times with different inocula. (iii) The reproduction patterns from single cells and the resultant cell groupings were consistent with those normally found (9).

TABLE 3. *Percentages of culture losses and detection of contaminants in apparently pure cultures[a] at each transfer number of B. matruchotii primary isolates under aerobic culture on BHI*

| Class | Transfer no. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4-6 | 7 | 8 | 9 | 12 | >14 |
| Losses[b] | | | | | | | | | |
| Non-transferable (%) | 15 | 7 | 7 | 1 | 5 | 4 | 1 | <1 | 2 |
| Contaminant over-growth (%) | 0 | 1 | 1 | <1 | 2 | 0 | 0 | 2 | 0 |
| Apparently pure to contaminated (%)[b] | | | | | | 3 | 4 | 0 | 3 |

[a] 10% which were judged pure by routine morphological examinations at three consecutive transfers (no. 5-7) subsequently-were found to be contaminated with a microorganism present in the primary isolate.

[b] 50% were lost because of non-transferability or overgrowth by contaminants.

## DISCUSSION

The previously reported morphological variability in *B. matruchotii*, resulting from mode of reproduction, culture age, and cultivation condition, was confirmed, as was the occurrence of an R → I → S morphological dissociation. The data suggest that morphological dissociation occurs from mutant selection as reported for other systems (14). Primary isolates from the natural habitat of dental plaque or calculus generally form the distinctive R colonies, although S colonies may occur (2; B. G. Bibby, Ph.D. thesis, University of Rochester, Rochester, N.Y., 1935).

However, "morphological dissociation," wherein *B. matruchotii* was reported to give rise to a variety of genera and species (20; J. L. Streckfuss, W. N. Smith, A. C. Taylor, and J. Ennever, Int. Ass. Dent. Res. Abstr. no. 192, 1971; W. N. Smith, J. L. Streckfuss, and L. R. Brown, Int. Ass. Dent. Res. Abstr. no. 59, 1971) could not be confirmed. Although some aspects of the phenomenon were observed, these were attributable to pleomorphism and contaminant entry and, therefore, do not support the proposed extreme genetic instability for this microorganism. Further, to date there is no evidence which would allow attribution of the observations to endoparasitic bacterial symbionts.

The data presented, in conjunction with L-phase and *Mycoplasma* studies (3, 17), show that the establishment of pure cultures and the assessment of culture purity often present greater difficulties than the frequently casual treatment of them warrants. The isolation of a single cell by micromanipulation for culture initiation does not necessarily ensure purity of subsequent cultures; with inadequate optics smaller adherent heterologous microorganisms may not be detected. Contaminant entry routes are numerous, diverse (4), and often unrecognized (1, 3, 14), and it cannot be assumed that techniques for entry reduction are equivalent to

eradication (5, 8, 15). Moreover, growth initiation from a single cell is not attainable with some microorganisms (e.g., spirochetes), nor are micromanipulators and/or the technique for utilizing them available to all.

The criteria selected for culture purity assessment are therefore important. Cellular and colonial morphologies are used routinely. The former is unreliable when applied to cultures of microorganisms which have variable cellular morphologies or where the contaminant and the desired culture have similar morphologies. In either case, contaminant discrimination by routine means then relies solely upon colonial morphology differences. The latter is of limited value when cultivation conditions are suboptimal for the contaminant or when the technique employed is insensitive to low contaminant levels. These morphological characteristics are therefore often as inadequate for culture purity assessment, particularly with pleomorphic microorganisms, as they are recognized to be for classification purposes. Other characteristics must be employed in conjunction with them. These should include a minimum of two attributes which reflect different cellular properties in order to allow for the normal variability which will occur in the large populations being studied. For example, deoxyribonucleic acid assays may be affected by plasmid, prophage, or intracellular bacterial parasite content. Although purity assessments based upon reproducibility of major characteristics and consistent colonial and cellular morphologies under a variety of growth conditions will minimize errors, the successful use of these criteria remains dependent upon a judicious regard for systematic contaminant entry sources, technical limitations, and symbiotic relationships between mixtures and pleomorphism. However, with their proper application to cultures which appear to indicate extremely novel biological phenomena, confusion in the literature will be reduced. This same restraint also applies to reports of the isolation of novel microorganisms from mixed natural ecosystems, where purity is often assumed with the use of an isolated colony on the primary or secondary plate.

The criteria employed herein for *B. matruchotii*, namely morphological changes in response to growth conditions, mode of reproduction, and simple fermentative and biochemical characteristics, should not be regarded as either universally applicable or optimal. The first of these requires considerable experience with the microorganism, and the latter two would be susceptible to species variability. Other utilizable criteria include antigenic composition and deoxyribonucleic acid analyses. For *B. matruchotii*, the percentage of guanine plus cytosine (G+C) is 55 to 57 (L. R. Page and G. N. Krywolap, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1973, G101, p. 43) and 53 (B. F. Hammond, personal communication). In contrast, the G+C mole percent values ranged from 35 to 41 for the various subspecies of *Streptococcus mutans* (6), one of the reported "variants" isolated from *B. matruchotii*.

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## LITERATURE CITED

1. Barile, M. F., and R. A. Delguidise. 1972. Isolations of Mycoplasmas and their rapid identification by plate epi-immunofluorescence, p. 165-181. *In* Pathogenic mycoplasmas, CIBA Foundation Symposium. Elsevier, New York.
2. Bibby, B. G., and G. P. Berry. 1939. A cultural study of filamentous bacteria obtained from the human mouth. J. Bacteriol. **38**:263-274.
3. Clasener, H. 1972. Pathogenicity of the L-phase of bacteria. Annu. Rev. Microbiol. **26**:55-84.
4. Darlow, H. M. 1969. Safety in the microbiological laboratory, p. 169-203. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 1. Academic Press Inc., New York.
5. Doyle, J. E., and R. R. Ernst. 1969. Alcohol flaming—a possible source of contamination in sterility testing. Amer. J. Clin. Pathol. **51**:407-408.
6. Dunny, G. M., T. Hausner, and D. B. Clewell, 1972. Buoyant densities of DNA from various strains of *Streptococcus mutans*. Arch. Oral Biol. **17**:1001-1003.
7. Ennever, J., L. Streckfuss, and I. Takazoe. 1973. Calcification of bacillary and streptococcal variants of *Bacterionema matruchotii*. J. Dent. Res. **52**:305-308.
8. Ernst, R. R., and A. P. Kretz, Jr. 1964. Compatability of sterilization and contamination control with application to spacecraft assembly. J. Amer. Ass. Contam. Contr. **10**:3-7.
9. Gilmour, M. N. 1961. The classification of organisms termed *Leptotrichia* (*Leptothrix*) *buccalis*. II. Reproduction of *Bacterionema matruchotii*. Bacteriol. Rev. **25**:142-151.
10. Gilmour, M. N., and P. H. Beck. 1961. The classification of organisms termed *Leptotrichia* (*Leptothrix*) *buccalis*. III. Growth and biochemical characteristics of *Bacterionema matruchotii*. Bacteriol. Rev. **25**:152-161.
11. Gilmour, M. N., A. Howell, Jr., and B. G. Bibby. 1961. The classification of organisms termed *Leptotrichia* (*Leptothrix*) *buccalis*. I. Review of the literature and proposed separation into *Leptotrichia buccalis* Trevisan, 1879 and *Bacterionema* gen. nov., *B. matruchotii* (Mendel, 1919) comb. nov. Bacteriol. Rev. **25**:131-141.
12. Gilmour, M. N., and P. A. Hunter, 1958. Isolation of an oral filamentous microorganism. J. Bacteriol. **76**:294-300.
13. Gilmour, M. N., and A. E. Poole. 1970. Growth stimula-

tion of the mixed microbial flora of human dental plaques by haemin. Arch. Oral Biol. **15**:1343–1353.
14. Lamanna, C., M. F. Mallette, and L. N. Zimmerman. 1973. Basic bacteriology, p. 620–624, 4th ed. The Williams & Wilkins Co., Baltimore.
15. McDade, J. J., G. B. Phillips, H. D. Sivinski, and W. J. Whitfield. 1969. Principles and applications of laminar-flow devices, p. 137–168. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 1. Academic Press Inc., New York.
16. Morris, E. O. 1954. The bacteriology of the oral cavity. V. Corynebacterium and gram-positive filamentous organisms. Brit. Dent. J. **97**:30–36.
17. Pirie, N. W. 1972. Introduction: principle of mini-life. p. 1–15. *In* Pathogenic mycoplasmas, CIBA Foundation Symposium. Elsevier, New York.
18. Richardson, R. L., and Schmidt, J. 1959. An oral filamentous microorganism: cultural characteristics and microbial relationships affecting growth. J. Dent. Res. **38**:1016–1027.
19. Smith, W., J. Streckfuss, J. Vogel, and J. Ennever. 1971. Struvite crystals in colonies of *Bacterionema matruchotii* and its variants. J. Dent. Res. **50**:777.
20. Streckfuss, J. L., and W. N. Smith. 1970. Isolation of bacillary and streptococcal variants from *Bacterionema matruchotii*. J. Bacteriol. **104**:1399–1400.