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Mode of Filamentous Growth of *Leucothrix mucor* in Pure Culture and in Nature, as Studied by Tritiated Thymidine Autoradiography

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Mode of growth of *Leucothrix mucor* filaments was measured by autoradiography with tritiated thymidine. Studies were performed on *L. mucor* in pure cultures in free suspension, as an epiphyte of pure cultures of the red alga *Antithamnion sarniense*, and as an epiphyte of red algae in the sea. Statistical analyses of the distribution of growing cells was done by use of the nonparametric One-Sample Runs Test and a Cluster analysis adapted from quadrat analyses of plant ecologists. No evidence of preferential growth at base or tip of *L. mucor* filaments was obtained in any of these studies. However, in nature, but not in the laboratory, there were regions of *L. mucor* filaments which were nongrowing or dormant. Such nongrowing regions could incorporate tritiated glucose.

Leucothrix mucor is a filamentous bacterium which lives in nature as an epiphyte of marine algae (3). A *Leucothrix* filament is attached at one end by a specific holdfast to the algal surface, and the filament grows out perpendicularly into the surrounding water. *L. mucor* can also be grown epiphytically in two-member culture with axenic marine algae (3), or it can be grown in pure culture in seawater to which organic energy sources are added (8). Under certain conditions, the cells of *L. mucor* filaments convert into spherical cells called gonidia which break off from the filament, settle on surfaces, and exhibit gliding motility. Harold and Stanier (8) observed this process in some detail, and described what they term the basal-apical differentiation of filaments; gonidia were observed by them to form preferentially at the apex of filaments. Since *L. mucor* is an epiphyte it seemed that differences might exist between base and apex in growth rate. It was assumed that epiphytic growth would occur predominantly at the base of a *L. mucor* filament, since the base would be nearest the source of organic nutrients (the algal frond). Mode of growth was thus studied by tritiated thymidine autoradiography, since this provided a direct means of observing which cells in a filament were growing (5). This technique has also been used with *L. mucor* to study the rate of growth in epiphytic situations (4). As will be seen, the hypothesis of preferential basal growth has turned out to be wrong, and the data neither support nor refute the idea of basal-apical differentiation unrelated to cell division.

MATERIALS AND METHODS

The validity of the technique of tritiated thymidine autoradiography for *L. mucor* has been described elsewhere, as have the cultures, culture media, and methods (1, 3, 6). The culture of the marine alga *Antithamnion sarniense* was also described previously (3). Briefly, it was cultured in medium ASP_6 of Provasoli (10), which is a synthetic seawater medium buffered with tris(hydroxymethyl)aminomethane. This medium contains vitamins, trace elements, and other growth factors, but its organic content is such that *L. mucor* will not grow in it. The alga was grown at 15 C at 1,000 lux illumination in an alternating cycle of 14-hr light and 10-hr dark, in 16-mm screw-capped tubes containing 1 to 2 ml of medium. Two-membered cultures were prepared by inoculating 2-week-old algal cultures by loop from a 2-day culture of *L. mucor* which contained many gonidia. Within 3 days, the *L. mucor* had attached to the alga and was growing extensively. Such two-membered cultures have been periodically transferred to fresh medium and have been maintained for about 2 years. Exposure to tritiated thymidine (1μc/ml, 6.7 c/mmole) was generally less than 1 hr, and exposure was terminated by adding 0.1 volume of 38% formaldehyde. Material was affixed to slides with the aid of Ullrich's adhesive, and the slides were washed several times in 5% trichloroacetic acid followed by distilled water. Slides were dipped in Kodak NTB-2 liquid emulsion (diluted 1:2.5), allowed to expose for 3 to 7 days, and de-

veloped. Microscopic analysis of autoradiograms was with a Carl Zeiss phase microscope with oil immersion lens (numerical aperture, 1.32). 6^3H-glucose (487 mc/mmole) was also used at a final concentration of 1 μc/ml.

Well-isolated *L. mucor* filaments were analyzed quantitatively by scoring each cell of a filament as to whether or not it was radioactive, as revealed by the presence above it of a silver grain. Cells with more than one silver grain were not differentiated from those with only one grain. The data thus obtained are the distributions of radioactive cells along the length of a filament, from base (where attached to the algal frond) to tip. Rate of accumulation of radioactive cells was linear with time until 80% of the cells were radioactive, and was proportional to growth rate (4).

Autoradiograms were prepared from three kinds of materials: (i) pure cultures growing logarithmically in free suspension in glutamate-synthetic seawater medium (3); (ii) pure cultures growing epiphytically on pure cultures of the red alga *Antithamnion sarniense;* (iii) mixed natural cultures growing in the sea on various filamentous algae.

RESULTS

The data from a large number of individual filaments of varying length were analyzed statistically to determine whether growth was localized in any particular region of a filament. Short incubation times were used, in which less than 25 to 30% of all the cells in a filament were labeled; long incubation times were also used, in which more than 75 to 80% of the cells in a filament were labeled. By merely dividing each filament in half and calculating the percentage of cells in each half which were radioactive, it could be readily shown that there was no localization of growth at one end or the other. Although, for a given

TABLE 1. *Counts on free-living Leucothrix mucor filaments*[a]

Position of cell within a filament	Labeling time, 5 min			Labeling time, 30 min		
	No. of cells radioactive	Total no. of cells in this position	Per cent radioactive in this position	No. of cells radioactive	Total no. of cells in this position	Per cent radioactive in this position
1	29	99	29	78	100	78
2	39	99	39	65	100	65
3	26	99	26	70	100	70
4	37	96	39	70	95	74
5	28	86	33	62	84	76
6	23	76	30	56	72	78
7	30	65	46	43	64	67
8	20	60	33	45	58	78
9	18	52	35	39	53	74
10	11	36	31	35	48	73
11	16	43	37	31	114	70
12	13	35	37	29	40	72
13	10	32	31	29	36	80
14	7	28	25	20	30	67
15	9	24	38	18	26	69

[a] With 5-min labeling, 99 separate filaments were counted; length, 3 to 15 cells. With 30-min labeling, 100 separate filaments were counted; length, 3 to 15 cells. Medium: 1% glutamate-synthetic seawater, 25 C.

TABLE 2. *Counts of Leucothrix mucor filaments growing epiphytically on Antithamnion sarniense*[a]

Position of cell within a filament	No. of cells radioactive	Total no. of cells in this position	Per cent radioactive in this position
1 (base)	23	42	55
2	21	42	50
3	22	42	52
4	22	42	52
5	21	40	53
6	20	37	54
7	22	34	65
8	17	33	52
9	16	31	52
10	16	27	59
11	19	26	73
12	12	24	50
13	9	22	41
14	16	22	73
15	9	20	45
16	11	20	55
17	12	19	63
18	13	18	72

[a] Forty-two separate filaments were counted. Filament length varied from 3 to 18 cells. Medium: ASP_6 (10), 15 C. Labeling time, 5 min.

TABLE 3. *Data from the filament illustrated in Fig. 1*[a]

Sample size	Mean of radioactive cells per sample	Variance	Variance/mean	Conclusion
4	1.7	1.9	1.12	Nonrandom?
6	3.0	2.7	0.9	Random
8	4.0	1.0	0.25	Random
10	4.5	12.5	2.78	Nonrandom
12	6.0	8.0	1.33	Nonrandom
14	6.0	18.0	3.0	Nonrandom

[a] As shown in Fig. 1, successive samples of size 4 have 2, 3, 3, 1, 0, 3, and 0 radioactive cells per sample. With 7 samples, mean is 12/7 or 1.7. Variance calculated by standard statistical methods. Mean number of radioactive cells per sample is calculated for sample sizes of 4, 6, 8, 10, 12, and 14 cells per sample. Variance and variance-mean ratio are then calculated. If the ratio is greater than 1, then clustering is indicated for this sample size.

filament, one half or the other might be more radioactive, average values for large numbers of filaments showed no evidence of basal or apical localization of dividing cells, either in pure cultures growing in free suspension or in epiphytically growing organisms. In addition, the percentage of cells at each position along the filaments was also calculated, and representative data are given in Table 1 (for free-living cultures) and Table 2 (for epiphytically growing cultures). Again, no evidence of basal-apical growth differences was seen. Although most of the filaments analyzed were fairly short, several very long filaments (not shown in tables), with lengths of 110, 110, 131, 183, and 702 cells per filament, were also studied. Even in such long filaments, there was no evidence of preferential regions of growth.

FIG. 1. *Diagrammatic representation of procedure for cluster analysis.*

To determine whether clustering of a more localized nature might occur, two kinds of statistical analyses were used. The first of these was the One-Sample Runs Test (11), a simple nonparametric test which indicated merely whether or not cluster-

TABLE 4. *Statistical analysis of distribution of cell division in epiphytic filaments of Leucothrix mucor*[a]

Filament no.	No. of cells/filament	Per cent of cells radioactive		One-Sample Runs Test *Z* value	Cluster analysis (no. of cells per cluster)
		Tip half	Base half		
1	71	22.5	15.5	2.41	12, 14
2	39	38	10	3.28	4, 6, 8, 10, 16, 18
3	42	29	21	0.937	10, 16
4	45	42	29	1.66	12
5	38	26	26	2.62	6, 8, 12
6	45	11	31	1.56	4, 12, 18, 20, 22
7	63	9.5	21	2.78	6, 8, 10, 12, 14, 16, 18, 20, 22
8	43	16	28	1.95	8, 12, 16, 18, 20
9	35	20	20	1.72	4, 16
10	45	27	9	1.86	6, 8, 10, 12
11	65	23	28	2.37	8, 10, 20
12	53	26	23	2.64	4, 8, 10
13	116	28	34	3.09	20, 30
14	44	14	14	0.95	None
15	54	13	30	1.24	8, 10, 12, 14, 16, 18, 20, 22
16	73	33	26	3.44	8
17	121	21	20	4.23	18, 20
18	94	28	19	3.42	4
19	64	16	20	1.50	8, 10, 14, 18, 20
20	40	10	20	0.46	14, 16, 20
21	65	31	22	2.27	8, 10, 12, 14, 16, 18
22	52	31	17	0.24	None
23	52	10	33	2.41	6, 8, 10, 12, 14, 16, 18, 20
24	98	29	15	3.77	14, 16, 18, 30
25	16	31	37.5	0.67	None
26	26	7.7	27	1.23	4, 12
27	24	17	8	0.565	4
28	14	7	0	4.73	4
29	28	32	11	0.71	4, 10, 12, 14
30	20	25	35	2.22	4
31	34	29	21	0.77	4, 10, 12
32	26	19	15	3.42	6
33	25	20	20	0.855	None
Avg		22.5	21.4		

[a] Analysis done on samples of red alga from Sudurnes, Iceland, incubated for 1 hr with 1 μc/ml of tritiated thymidine at ambient temperature, 13.2 C, 4 August 1965, 5:00 to 6:00 PM. See Brock (4) for actual data. If the *Z* value of the One-Sample Runs Test is greater than 1.96, distribution is nonrandom at the 5% level of significance (10). Cluster analysis performed as described in text. Values listed are numbers of cells per cluster.

ing occurred, but not the nature of the clusters. Nonrandom distribution would be indicated by a Z value greater than 1.96 (5% level of significance). Although this test was relatively simple to perform on a large number of filaments, it did not indicate the sizes of the clusters, and it also led to erroneous conclusions in those cases where most of a filament showed random growth but a single region showed clustering. A more detailed analysis was obtained by modifying a statistical test used by plant ecologists to detect pattern or nonrandomness in quadrat analyses (7, 9). As shown by plant ecologists, nonrandomness can be detected if counts are made in the same area divided into quadrats of different sizes, and the variance-mean ratio for each quadrat size is calculated. For nonrandom or clustered areas, the variance-mean ratio is greater than 1.0 for certain quadrat sizes. In the present modification of this technique, a filament was divided successively into groups of 4, 6, 8, 10, 12, 14, 16, 18, 20, and 30 cells, and the mean and variance of the number of radioactive cells in each sample size was determined (Table 3). The use of this procedure is illustrated in Fig. 1.

When both kinds of analyses were run on autoradiograms from pure cultures, there was no evidence of any small regions within a filament where cell division was concentrated. This was true both for short filaments (less than 20 cells) and very long filaments (over 700 cells). Thus, it can be concluded that in pure cultures, under the conditions used, cell division is randomly distributed along the filament. It might be noted in passing that the cells in regions of *L. mucor* filaments forming knots (1) also became labeled, thus confirming the previous suggestion that knot formation is the result of a growth process. Label was also seen in gonidia, as well as in cells in filaments which seemed in the process of releasing gonidia. The only cellular structure in *L. mucor* which did not become labeled was the bulb (1), and other unpublished data suggest that this structure may not be capable of growth.

With pure cultures of *L. mucor* growing epiphytically on *A. sarniense*, only 7% of the filaments showed evidence of clustered growth, and 93% of the filaments showed completely random growth, by the One-Sample Runs Test. It might also be noted that growth rate of *L. mucor* on *A. sarniense* was even faster than in pure culture (4).

In contrast, in natural material, clustered growth was found very frequently. Three series of experiments done in nature have been analyzed in detail: (i) late April 1965, at Long Island Sound and Narragansett Bay; (ii) mid-July 1965, at Narragansett Bay; (iii) late July and early August 1965, at Cape Reykjanes and Sudurnes near Reykjavik, Iceland. Various filamentous algae and several incubation times were used, but, because of the slower division rates in nature, only the longer incubation times (60 min) provided a sufficient percentage of radioactive cells for suitable analysis. One-Sample Runs Tests showed that the distribution of radioactive cells in many cases was nonrandom (data from a representative set are shown in Table 4). However, the One-Sample Runs Test is not efficient for detecting isolated clustered regions within a filament which otherwise shows random growth. The more laborious cluster analysis described above was thus carried out on all the filaments shown in Table 4. It can be seen that radioactive cells occurred in clusters of four to eight or more cells, interspaced with clusters of nonradioactive cells. Only 3 of 33 filaments did not show clustered regions. Similar results were obtained in a variety of other samples examined. A photomicrograph of such radioactive clusters can be seen in Fig. 2. These results contrast with those of the pure and two-membered cultures, in which only random growth was seen,

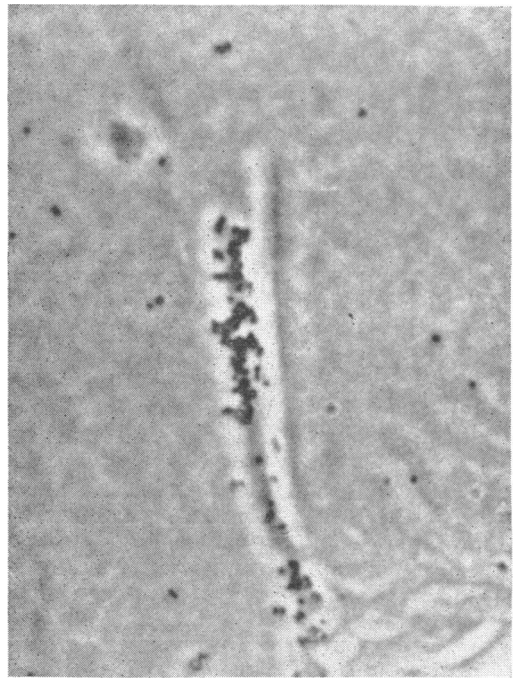

FIG. 2. *Clustered growth of short-term incubation. Autoradiogram of Leucothrix mucor filament growing on a filamentous brown alga. Quahaug Rock, Narragansett Bay, 2 May 1965. Water temperature, 12 C. Incubation time with ^{3}H-thymidine, 60 min (3:00 to 4:00 PM). Autoradiogram exposure, 7 days. Phase contrast, × 1,333.*

whether analyzed by the One-Sample Runs Test or by cluster analysis.

In one set of samples (from Long Island Sound), the incubation with tritiated thymidine was allowed to continue throughout the night, for a total incubation time of 18 hr. When these samples were examined autoradiographically, it was found that although the majority of the cells were heavily radioactive (too heavy to allow quantitative study) there were occasional regions along a filament which were completely nonradioactive (Fig. 3). Thus, it seems likely that in nature certain regions of a filament may not be growing, whereas the rest of the filament is growing. It should be emphasized that even in nature there was no evidence of preferential growth at either base or apex, but only of the existence of small nongrowing regions.

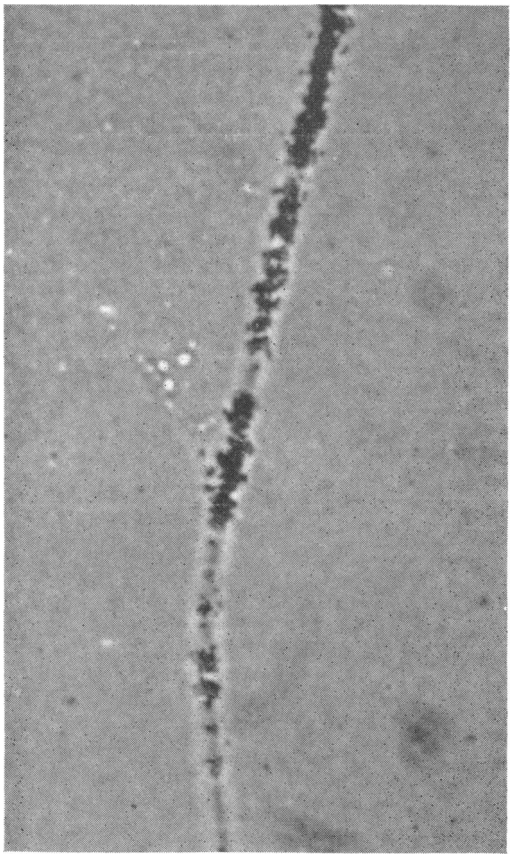

FIG. 3. *Clustered growth of long-term incubation. Autoradiogram of Leucothrix mucor filament growing on filamentous red alga, Long Island Sound, Woodmont, Conn., 30 April 1965. Water temperature, 9 C. Incubation time with ^{3}H-thymidine, 18 hr (4:00 PM to 10:00 AM). Autoradiogram exposure, 7 days. Phase contrast, × 1,333.*

TABLE 5. *Incorporation of ^{3}H-glucose: statistical analysis by One-Sample Runs Test*[a]

Filament no.	No. of cells/filament	*Z* value
1	59	.032
2	45	.986
3	64	.107
4	73	.418
5	62	.664
6	79	.455
7	55	.558
8	73	.677
9	89	.002
10	30	.238
11	79	.578
12	72	.320
13	62	.014
14	64	.043
15	31	.226
16	34	.589
17	50	.250
18	40	.083
19	66	.726

[a] Analysis done on same alga as in Table 3, incubated for 1 hr with 1 μc/ml of tritiated glucose at 13.2 C, 4 August 1965, 5:00–6:00 PM. If the *Z* values were greater than 1.96, distribution would have been nonrandom (5% level of significance).

L. mucor can utilize glucose as a carbon source, and some experiments were done with tritiated glucose instead of tritiated thymidine. Although incorporation was excellent, no evidence of clustering was found when the data were analyzed statistically (Table 5). Thus, the regions of *L. mucor* filaments which in nature are not dividing are still able to incorporate glucose into macromolecules.

DISCUSSION

The present work shows that, in actively growing pure cultures, all of the cells in a *L. mucor* filament are able to divide. There is no evidence of differential cell division at base or apex, and it is appropriate to look at an *L. mucor* filament as a long collection of autonomous cells. This conclusion is in agreement with the fact that, in free suspension culture, *L. mucor* grows logarithmically (4), showing that each cell is able to give rise to offspring. However, basal-apical differentiation may exist for the process of gonidia formation, as first described by Harold and Stanier (8). Gonidia incorporate tritiated thymidine (4), and thus it would not be possible to detect localized regions of incipient gonidia formation, by the present methods.

Growth presumably occurs only when appropriate nutrients are present, and it is likely that the epiphytic bacteria obtain their nutrients either directly or indirectly from the algae. In the case of the laboratory culture of *Leucothrix* on *Antithamnion*, *Leucothrix* cannot grow alone in the medium (ASP_6) in which the alga grows, but grows only when attached to the alga. If nutrients pass directly to the bacteria from the algae without first being released into the water, then it would seem likely that the region of a bacterial filament closest to the algal frond should be growing most rapidly, since it would be in the presence of the highest nutrient concentration. On the other hand, if the algae release nutrients into the water, and complete mixing occurs, then there should be no nutrient gradient. All regions of a bacterial filament should have equal access to the nutrient supply, and there should be no preferential growth at the base. The present results support the latter alternative and suggest that in nature the epiphytic bacteria derive their nutrients from the seawater. This conclusion is of considerable significance for an attempt to analyze transfer of organic carbon from algae to bacteria, since it suggests that at least some of these epiphytes do not have any preferential access to the organic energy in comparison with free-living bacteria or other organisms. However, further work will be necessary to ascertain whether this same phenomenon occurs in other natural habitats and for other organisms. It should be recalled that the natural habitats studied were in areas of turbulent, well-mixed water, where nutrient gradients would be less likely to develop. In more quiet waters, the situation might be considerably different.

The clustering or nonrandom growth observed in natural material, but not in pure cultures or two-membered cultures, cannot be explained at present. Since it was observed in locations as diverse as Long Island Sound and Iceland, it cannot be considered an isolated phenomenon. It is clear that certain regions of *L. mucor* filaments are slow-growing or dormant, but the cause and outcome of this dormancy is unknown. This finding emphasizes the point often made (2) that laboratory studies cannot be automatically applied to nature. However, nonrandom labeling is not seen if ^3H-glucose is used instead of ^3H-thymidine, which shows that these unusual regions of a filament are still able to assimilate carbon sources.

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