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Morphological Study of the Reversion to Bacillary Form of *Bacillus megaterium* Protoplasts

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Protoplasts of *Bacillus megaterium* readily reverted to bacillary form in liquid media and when plated in a soft-agar layer onto the surface of appropriate agar media. Three phases of the reversion sequence could be differentiated by phase contrast microscopy: (i) increase in size of the individual protoplasts, (ii) non-oriented division of the protoplasts, and (iii) outgrowth of the bacillary forms. With time-lapse photomicrography, reversion sequences of single protoplasts were demonstrated.

Bacteria of *Bacillus megaterium* treated with the peptidoglycan-hydrolyzing enzyme lysozyme are transformed, in media of high osmolarity, to spherical bodies called protoplasts (10). These protoplasts maintain the ability to grow and divide in liquid media (1, 3, 5–7). It was recently reported that, when protoplasts of *B. megaterium* were plated in a soft-agar layer onto the surface of appropriate agar media, reverting bacillary forms appeared with high frequency within 48 h (2).

In this communication, morphological details of the reversion process of *B. megaterium* protoplasts are described, and photomicrographs demonstrate how single protoplasts in situ revert to the bacillary form.

MATERIALS AND METHODS

Strains. Strains used included *B. megaterium* KM, Leu⁻ and *B. megaterium* KM, Leu⁻, Arg⁻.

Media. The compositions of protoplasting and hypertonic media were described earlier (2). In some cases, to accelerate the process of reversion, 0.1% yeast extract (Difco) was included in the media.

Protoplasts. Protoplasts were produced as before (2). The term "protoplast" is used in our studies in its general meaning, i.e., spherical bodies, produced from bacilli of *B. megaterium* by lysozyme treatment in media with high osmolarity, which are disrupted at normal osmolarity.

Photomicrography. Morphology of the reverting protoplasts was observed by taking photographs in a Zeiss Amplival microscope with phase contrast illumination. The samples were studied by the following methods. (i) Samples were removed at intervals from the soft-agar layer, placed on a microscope slide, covered by a glass cover slip, and examined under an oil immersion objective. (ii) The setup seen in Fig. 1A was used for in situ photomicrography at low magnification (×400). (iii) The setup seen in Fig. 1B was used for in situ photomicrography at high magnification (oil immersion objective) (×1,600).

RESULTS

Incubation of the protoplasts in liquid media. The protoplasts were very stable in the liquid hypertonic medium. In static suspensions at room temperature, they were not disrupted for 2 to 3 days. Under these conditions, the size of the majority of protoplasts did not increase and reversion to bacillary forms was not observed.

When aeration was provided by gentle shaking of the protoplast suspension, considerable increase in the size of the individual protoplasts was observed and division was apparent after 24 h. Nevertheless, bacillary forms did not appear.

On the other hand, many bacillary forms were found after 24 h in an aerated liquid hypertonic medium layered over a solidified bottom layer of agar. In these experiments, the liquid layer was 1 to 2 ml, surmounting a 1% hypertonic agar (Difco) layer in the bottom of an Erlenmeyer flask, gently shaken at 35 C.

Incubation of the protoplasts in soft-agar media. When the hypertonic liquid medium contained 0.1 to 0.5% agar, the stability of protoplasts was increased, and they remained intact for 5 to 7 days at room temperature (19 to 21 C) in static cultures. In this medium, the size of 5 to 10% of the protoplasts increased significantly, and after 12 h of incubation, protoplast division was observed. No reverting bacillary forms appeared, however, under these conditions.

When the protoplast suspension in a soft-agar medium was poured onto the surface of the 1% agar layer in Erlenmeyer flasks, bacillary forms again appeared after 24 h of incubation whether the flasks were shaken or not.

Reverting bacillary forms also appeared with high frequency when the protoplast suspension

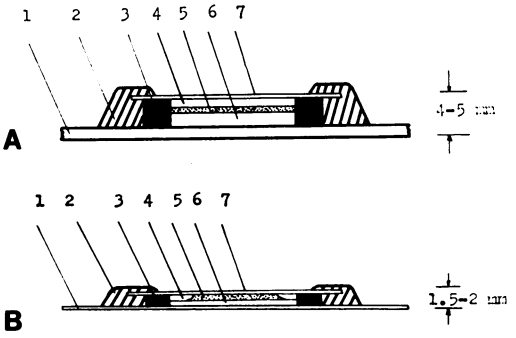

FIG. 1. *The setup for in situ photomicrography: (1) microscope slide (A) or cover slip (B); (2) beeswax; (3) cover holder; (4) aeration space; (5) soft-agar layer with the protoplasts; (6) bottom agar layer; and (7) cover slip.*

in hypertonic medium (0.1 to 0.25 M sucrose), containing 0.1 to 0.5% agar, was layered onto the surface of a 1% agar base layer (containing hypertonic medium) in petri dishes, and the plates were incubated in a wet chamber at 30 C (2). Subsequently, we found that incubation in a wet chamber was not obligatory, and the petri dishes were incubated in normal thermostats.

The stability of protoplasts in hypertonic media permitted us to dilute the suspensions and plate them in a soft-agar layer as individual clones, which, after reversion to bacillary forms, produced isolated bacterial colonies. Accordingly, by determining in a Coulter counter the number of protoplasts present in the suspension before plating, the reversion frequency of any protoplast suspension could be calculated.

The result of such an experiment is demonstrated in Table 1. Many similar experiments showed that 1 to 10% of the protoplasts of *B. megaterium* strain KM, Leu$^-$ and *B. megaterium* KM, Leu$^-$, Arg$^-$ were able to revert to bacillary forms.

Morphological changes of reverting protoplasts studied by phase contrast photomicrography. When samples were taken from the soft-agar layer at different time intervals after plating and studied by phase contrast microscopy, three phases could be differentiated as follows. (i) During the first few hours, samples contained only protoplasts, but of clearly increased size as shown in Fig. 2B. (ii) At 12 h, the picture was dominated by groups of dividing protoplasts (Fig. 2C and D). (iii) After 18 to 24 h of incubation, bacillary forms appeared (Fig. 3), and were seen more frequently with increased time.

The initial growth or swelling phase of this reversion sequence seemed to be a strict prerequisite for any further events. Increase in size of protoplasts was observed in numerous instances in liquid media by earlier workers (3, 5–7, 10) and confirmed in this study in aerated suspensions. Beyond this, in soft-agar layers we could regularly obtain reversion to the bacillary form.

Protoplast division, the second phase, was observed in the majority of experiments, and this may also be an essential step in the reversion sequence. In some cases, we were able to demonstrate that after only one or two divisions, bacilli were visible. Nevertheless, in the majority of cases many divisions preceded the appearance of rod forms.

Occasionally an alternative and unusual sequence of events was observed. After the initial phase, division of the enlarged protoplasts appeared to produce a morula-like structure of daughter protoplasts (Fig. 4). In this case, it appeared that each of the numerous daughter protoplasts reverted directly to a bacillary form.

In dense populations arising either by plating many protoplasts or by the division of protoplasts, aberrant division of the parent protoplasts occurred and the appearance of rod forms was very much delayed (Fig. 5). The irregular forms produced in some cases were very similar to those described by Fitz-James (1).

This morphological diversity in the second phase of the reversion sequence may depend upon many physiological and environmental factors, and we were unable to direct the sequences entirely at will by, for example, controlling the physiological state of bacteria used to produce protoplasts or by specific adjustments in the composition of our media.

TABLE 1. *Reversion frequency of a B. megaterium protoplast suspension*[a]

Dilution plated	Colony formers from:	
	Osmotically shocked suspension	Protoplast suspension
10^{-1}	No	Confluent growth
10^{-2}	–[b]	Confluent growth
10^{-3}	–	Confluent growth
10^{-4}	–	1,620
10^{-5}	–	230

[a] Strain, *B. megaterium* KM, Leu$^-$. The protoplast suspension contained 2×10^8 particles per ml as measured by a Coulter counter. One milliliter of the original suspension and of each dilution was mixed with 1 ml of 0.8% agar containing hypertonic medium and plated onto the surface of a hypertonic 1% agar medium.

[b] –, Not plated.

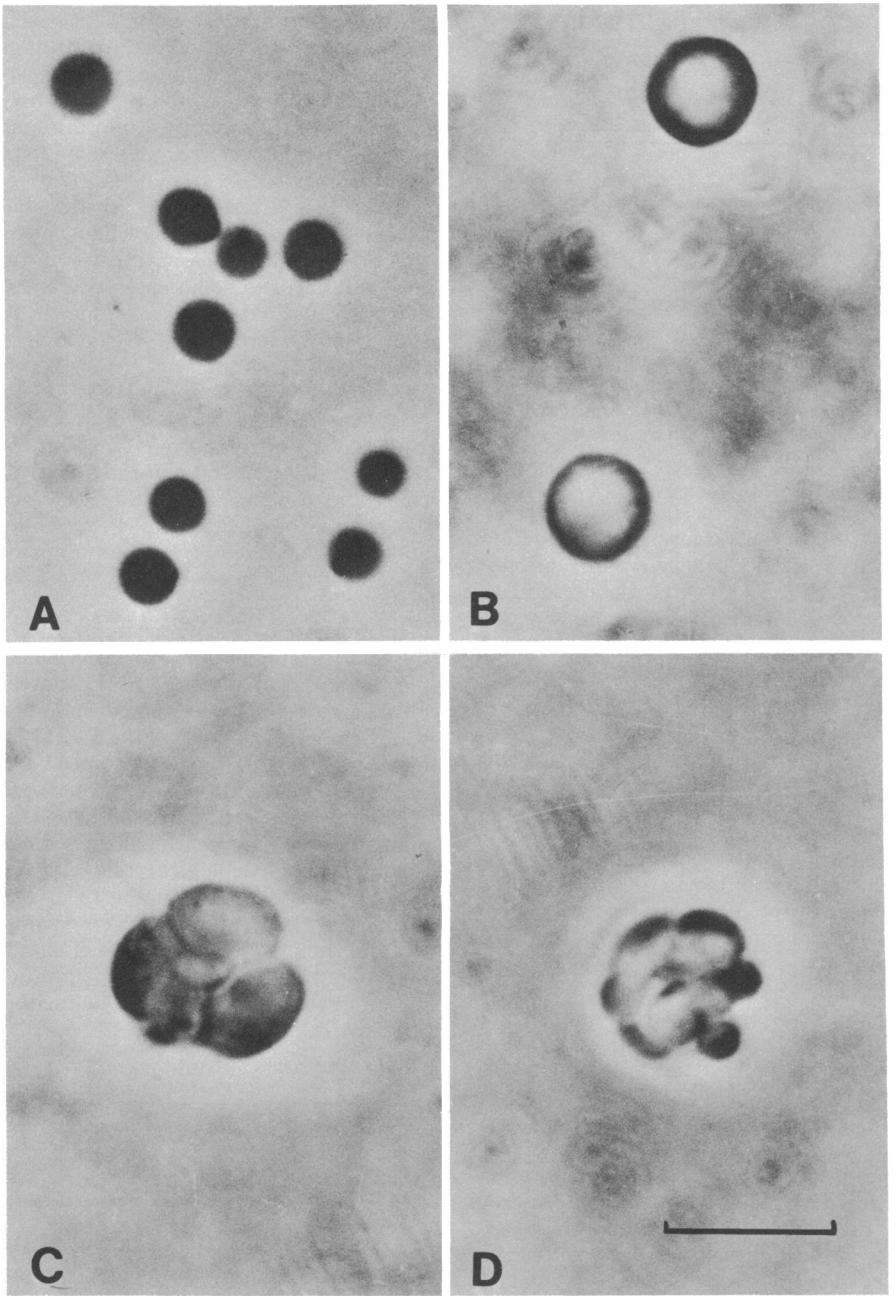

FIG. 2. *(A) Protoplast of B. megaterium KM, Leu⁻, Arg⁻; (B) enlarged protoplasts in the 0.25% soft-agar layer (6-h culture); (C) and (D) dividing protoplasts in the soft-agar layer (12-h culture). Bar represents 10 nm.*

In the third phase of the reversion sequence, bacillary forms appeared. The evidence presented above strongly suggested that these bacilli forms were generated directly from protoplasts. To confirm the clonal protoplast origin of these bacillary forms, attempts were made to follow at each stage the fate of a single protoplast. Because *B. megaterium* is a strict aerobe, the main difficulty was to supply sufficient oxygen under the cover slip throughout the whole reversion period.

The setup portrayed in Fig. 1B permitted

close-up photography of single protoplasts. Nevertheless, we were unable, after many attempts, to find a single protoplast that successfully completed the reversion sequence. The few protoplasts that divided did not proceed beyond this stage, presumably because of an inadequate supply of oxygen. This difficulty was finally overcome by preincubating the protoplasts in the predivision phase under well-aerated conditions and by imposing a certain synchrony on them by a cold treatment.

According to this procedure, 3 to 4 ml of the protoplast suspension was pipetted on the surface of a 1% agar-based (yeast extract-supple-

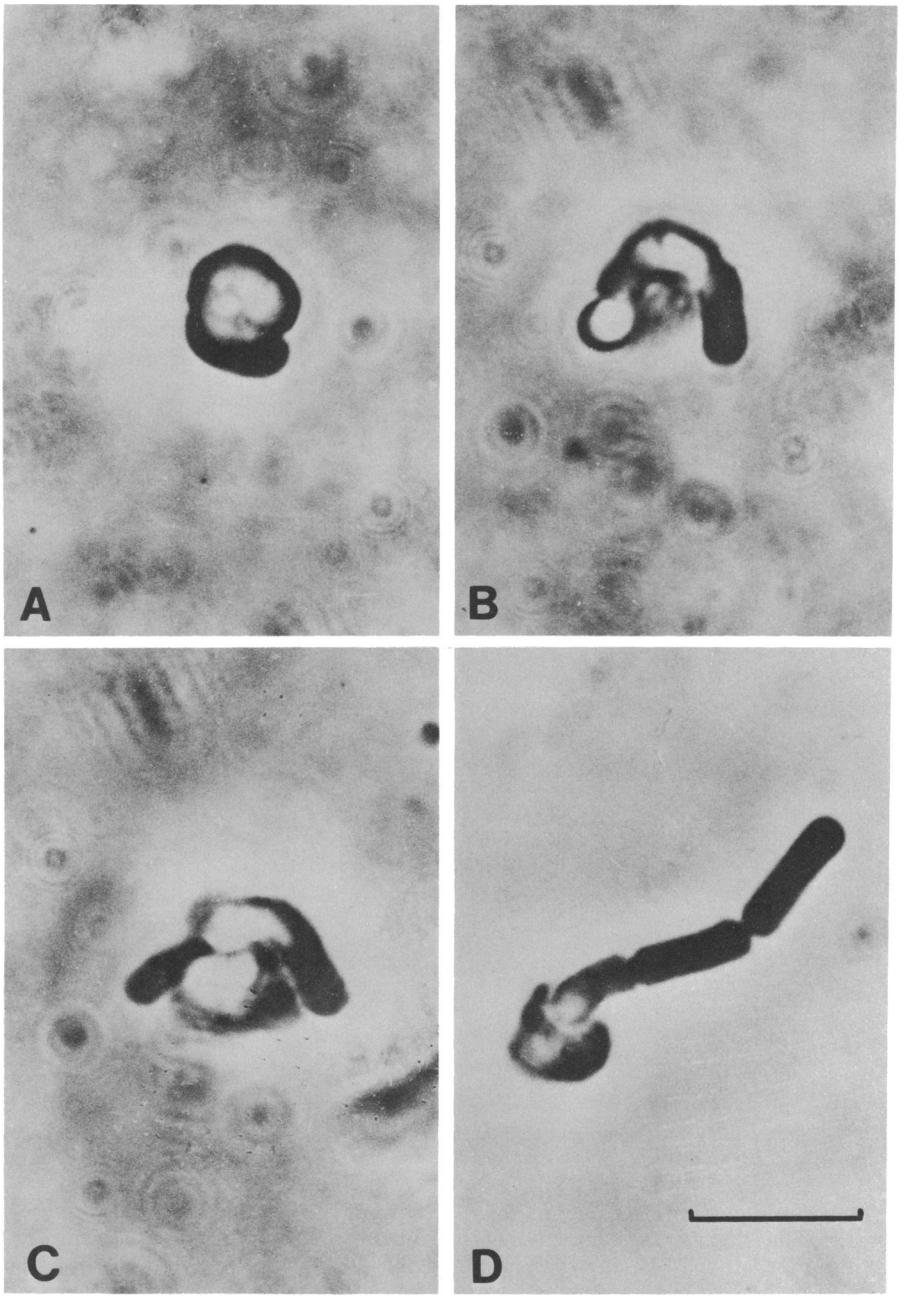

FIG. 3. *(A–D) Dividing protoplasts and outgrowth of bacillary forms in soft-agar layer; 24-h culture of the experiment of Fig. 2.*

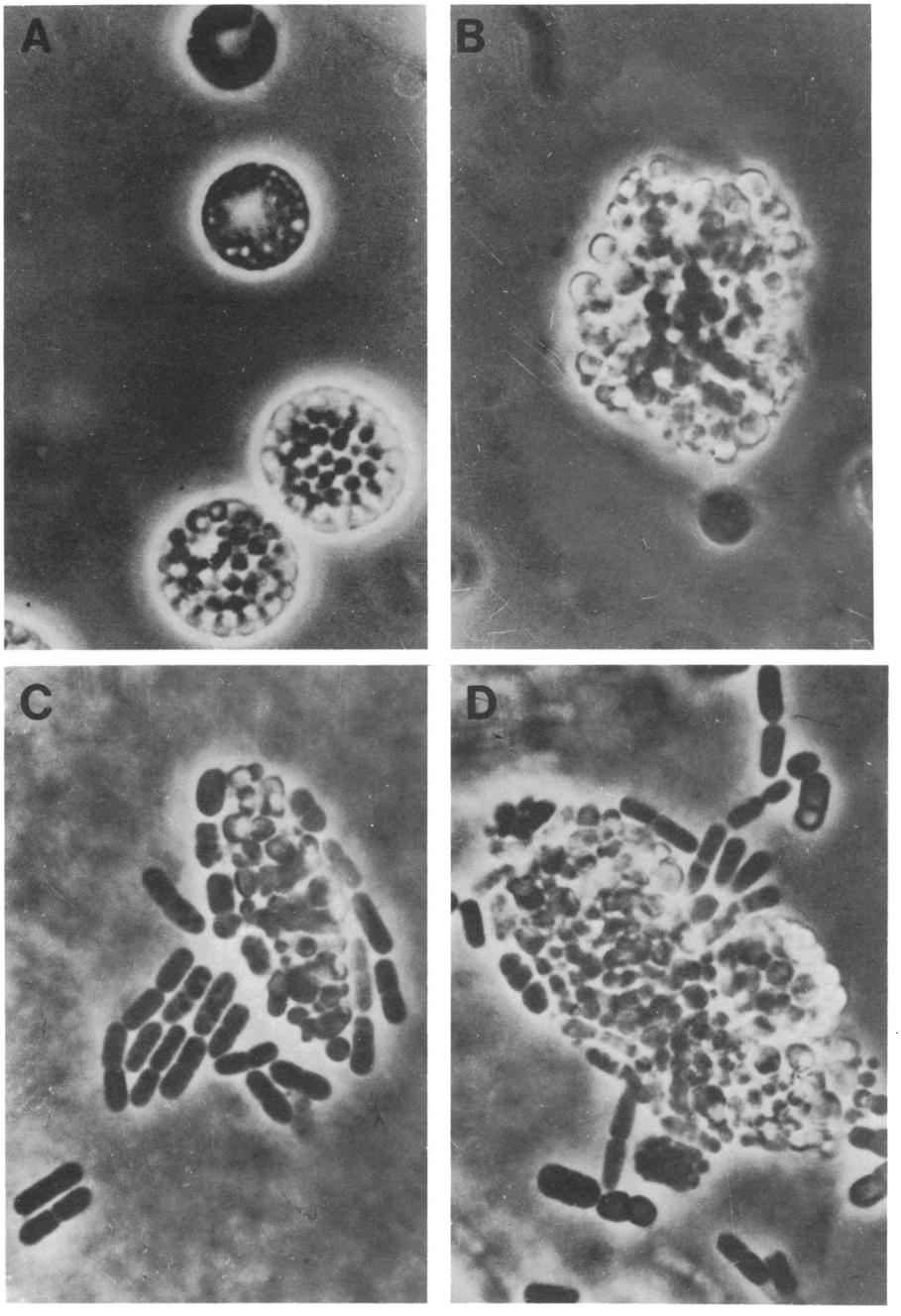

FIG. 4. *(A-D) Unusual sequence of reversion. Different phases of a possible reversion sequence found in the same sample of a 72-h-old culture. Strain: B. megaterium KM, Leu⁻.*

mented) hypertonic medium in a 100-ml Erlenmeyer flask. The flask was incubated with gentle shaking for 5 h at 25 C, and then at 6 C without shaking for 12 h. The protoplasts were then suspended at low concentration in a yeast extract-supplemented, hypertonic medium containing 0.25% agar and placed on the agar base in the cover slip setup shown in Fig. 1B. The microscope was kept at 25 C, and photographs were taken at various time intervals, zero time being when the microscopy examination started.

The photographs presented in Fig. 6 clearly demonstrate the in situ sequence of morphological steps that finally lead to the emergence of bacillary forms from an individual protoplast. These and other similar photographic sequences therefore removed any doubts about the clonal protoplast origin of the bacilli.

DISCUSSION

It has been a long-standing aim of many

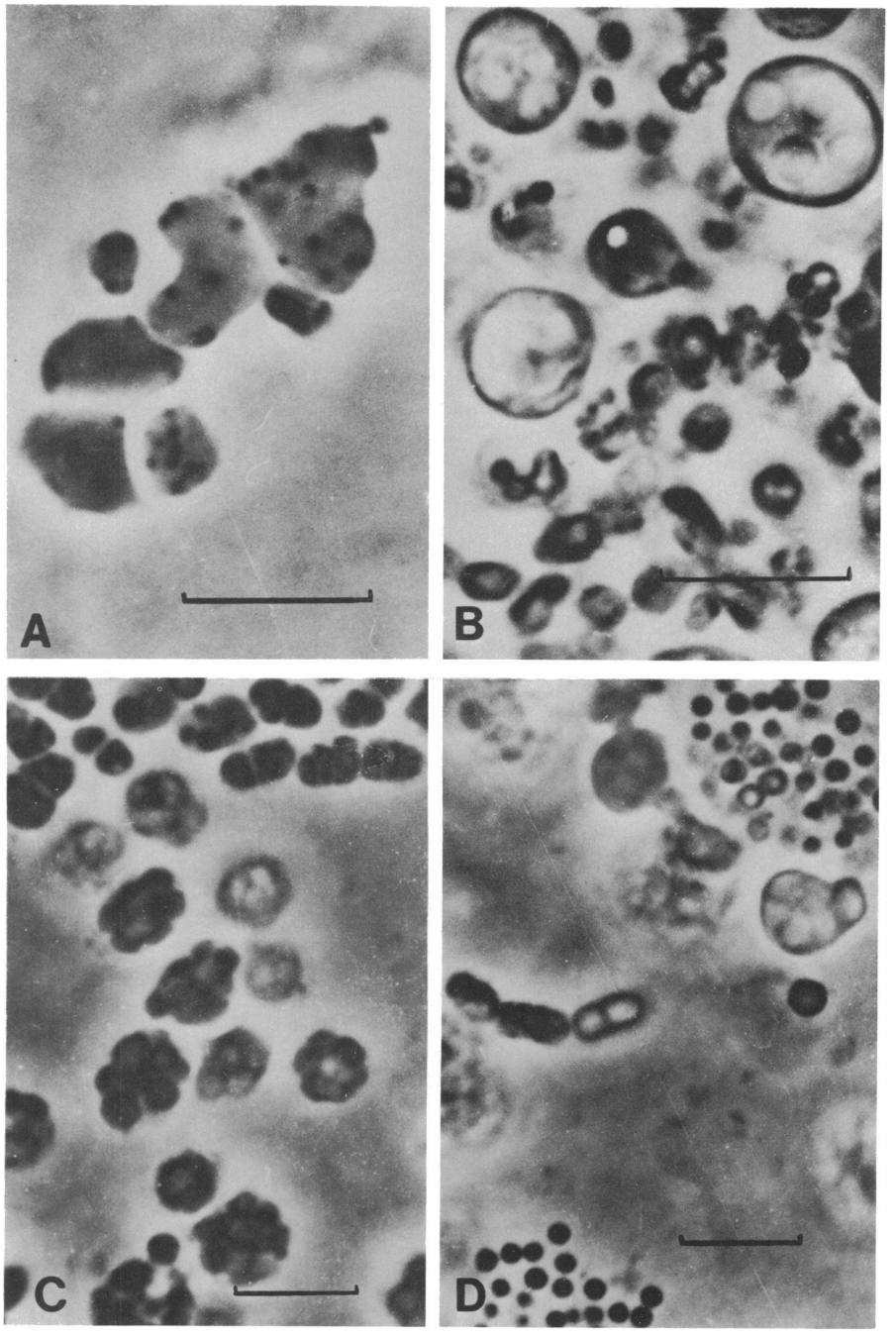

FIG. 5. *(A–D) Aberrant morphology arising from crowded protoplasts in soft-agar layer; 24- to 72-h culture.*

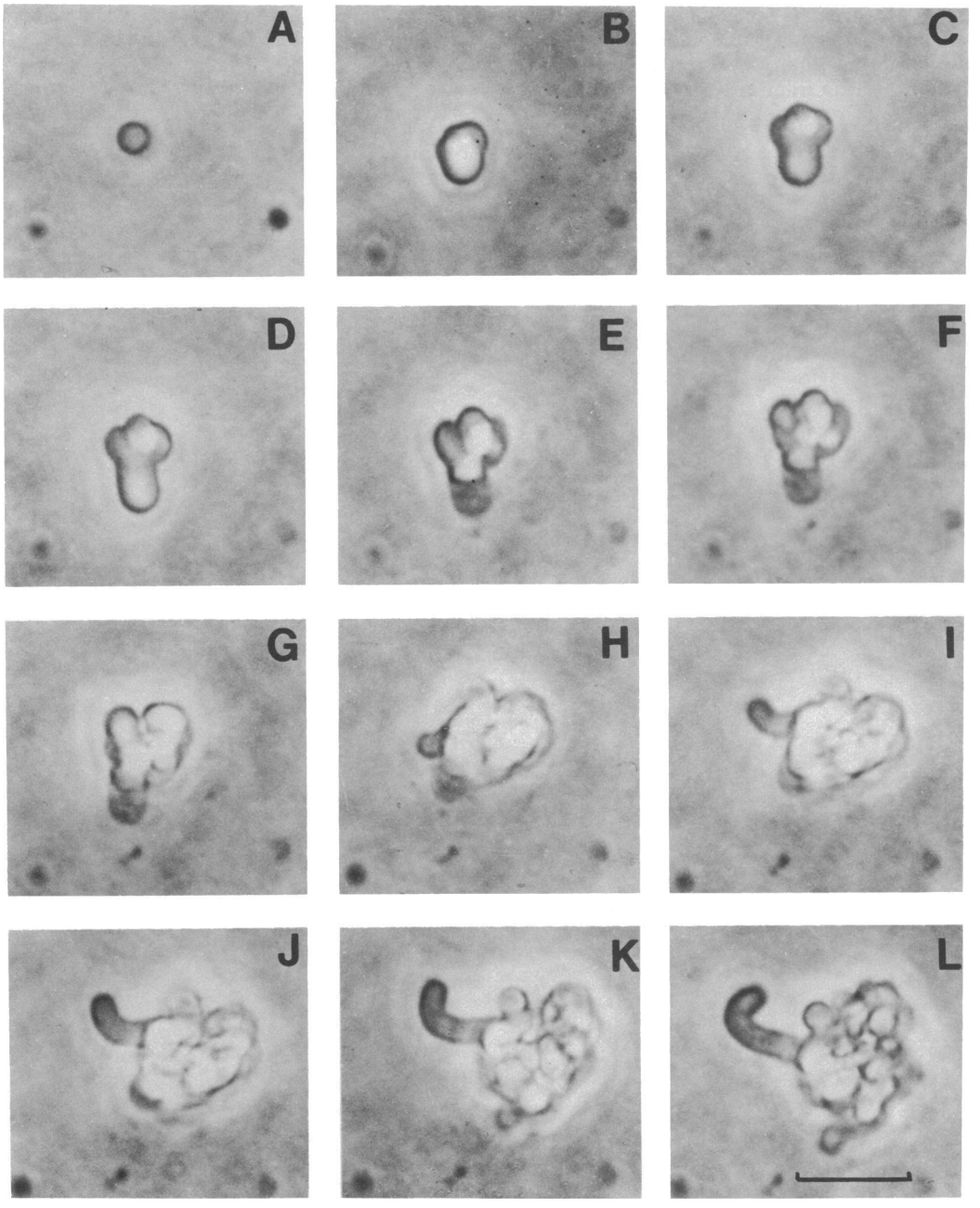

FIG. 6. *In situ time-lapse photomicrography of the reversion sequence of a single protoplast. High microscopic magnification. Oil immersion objective. Setup of the system as given in legend to Fig. 1B. (A) 0 h, (B) 6 h, (C) 6 h 30 min, (D) 7 h, (E) 7 h 30 min, (F) 8 h, (G) 8 h 30 min, (H) 9 h, (I) 9 h 10 min, (J) 9 h 20 min, (K) 9 h 30 min, (L) 9 h 40 min.*

studies with protoplasts of *B. megaterium* to find conditions in which bacillary forms might be regenerated. Previous studies by McQuillen (5–7), Fitz-James (1), and Kusaka (3) demonstrated that protoplasts of *B. megaterium* were able to grow and divide in liquid media, but reversion to bacillary form was not observed under these conditions. In a specific case, McQuillen (7) obtained a series of photomicrographs of a protoplast suspension in a micro-

drop culture hanging from a cover slip and observed in it "tangled masses of tubular growth." Nevertheless, he was cautious about the nature of this phenomenon, concluding that "It is, unfortunately, not known whether or not this represents reversion to bacillary form and cell wall formation."

We were able to confirm the findings of earlier workers, since in our hypertonic liquid medium in static culture reverting bacillary forms were never observed. We have also tested the effect of gelatin enrichment of liquid media, which induces reversion to bacillary forms of *B. subtilis* protoplasts (4), but this did not promote reversion of *B. megaterium* protoplasts (2).

In contrast, protoplasts of *B. megaterium* did produce bacillary forms when plated on solid media in a soft-agar layer (2), or when aerated in liquid suspension over such a layer.

In our earlier published photomicrographs, it seemed as though only a few linear division of protoplasts preceded the appearance of bacillary forms. In the photomicrographs presented here, however, different sequences of division and morphological changes having variable aspects can be seen before the appearance of the bacillary forms. In the majority of cases, the multiplication of the protoplasts seemed to be a multidirectional budding process rather than a linear division into equal parts. Some of our photomicrographs (Fig. 2 and 3) resemble those of *B. subtilis* reversion presented by Miller et al. (8), showing irregular forms undergoing a delayed conversion into rods. These authors (4, 8) did not observe replication under conditions they used until after the partially walled irregular bodies were produced. On the other hand, for *B. megaterium,* we present other pictures (Fig. 4) indicating a considerable multiplication of spherical forms, which then rather abruptly and completely grow out into rod forms.

When the local conditions are adequate for reversion, it can frequently be seen that all of the protoplasts in a microcolony become converted to bacilli at about the same time. This can be true even when other nearby microcolonies are not yet showing reversion.

The variation in the morphology of regenerating protoplasts could be explained by the sensitivity of the reversion process to environmental factors. Appearance of the first bacillary forms after a few divisions was obtained when protoplasts of early-logarithmic-phase bacteria were plated and the oxygen supply was abundant. Any change in growth conditions that reduced the oxygen supply (eg., plating greater concentrations of protoplasts) resulted in more protoplast division and in delayed appearance of the first bacillary forms. Clearly, adequate aeration of uncrowded populations appears to be one of the most important factors in the reversion process.

Slight changes of osmolarity may also significantly influence the reversion process. Although in some experiments varying the sucrose concentration between 0.1 and 0.25 M produced no effect, in other experiments early reversion was obtained at 0.1 and 0.15 M, and at 0.2 and 0.25 M sucrose the protoplast division phase persisted for a long time.

Finally, it is necessary to specify that the residual peptidoglycan content of the protoplast membrane was not measured in these studies. We are aware of the fact that the residual peptidoglycan content of these protoplasts might have varied, and this fact might also have influenced the reversion sequence. Furthermore, Reynolds, working on the in vitro peptidoglycan synthesis of *B. megaterium,* reports in one of his articles (9) that on the surface of a gelatine medium "reconditioned protoplasts" reverted to bacillary form.

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