Motility Mutants of *Dictyostelium discoideum*

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ABSTRACT We describe six motility mutants of *Dictyostelium discoideum* in this report. They were identified among a group of temperature-sensitive growth (Tsg) mutants that had been previously isolated using an enrichment for phagocytosis-defective cells. The Tsg mutants were screened for their ability to produce tracks on gold-coated cover slips, and several strains were found that were temperature-sensitive for migration in this assay. Analysis of spontaneous Tsg^+ revertants of 10 migration-defective strains identified six strains that co-reverted the Tsg and track formation phenotypes. Characterization of these six strains indicated that they were defective at restrictive temperature in track formation, phagocytosis of bacteria, and pseudopodial and filopodial activity, while retaining normal rates of oxygen consumption and viability. Because they had lost this group of motile capabilities, these strains were designated motility mutants. The Tsg^+ revertants of these mutants, which coordinately recovered all of the motile activities, were found at frequencies consistent with single genetic events. Analysis of the motility mutants and their revertants suggests a relationship between the motility mutations in some of these strains and genes affecting axenic growth.

Many aspects of motility in eucaryotic cells are thought to share a common molecular basis. Processes such as cell division, cytoplasmic streaming, endo- and exocytosis, and amoeboid motion all appear to involve an actomyosin-based mechanism. The evidence is mostly indirect, derived from morphological and ultrastructural studies and analysis of the properties of actin-rich cell extracts (reviewed in references 9 and 17).

A more direct approach to determining the role of specific components implicated in motility is through the use of genetic techniques. Lesions in specific proteins can provide a clear delineation of the roles played by those proteins. Several elements of the microtubule-based motility apparatus have been effectively studied in this manner (3, 4, 12, 16). The analysis of mutants can also reveal relationships between processes even without identification of the altered gene products. A group of activities dependent on the same component is indicated when all of the processes are altered by a single mutation; specific relationships within the group can be similarly distinguished (5, 15, 18, 20).

The eucaryotic microorganism *Dictyostelium discoideum* is a favorable system for combining biochemical and genetic approaches in analyzing motility. The amoebae have the same motile capabilities as cells of higher organisms and contain similar cytoskeletal proteins (for reviews, see references 7, 9, 17). They are haploid, facilitating the isolation of mutants, and a parasexual system permits genetic analysis (reviewed in reference 13).

As previously reported (6), a number of mutants of *D. discoideum* that are temperature-sensitive for growth on bacteria (Tsg) have been isolated by enriching for cells unable to phagocytose bacteria. The enrichment procedure used bromodeoxyuridine-containing bacteria followed by irradiation to kill amoebae that had incorporated bacterial nucleotide into their own DNA. Several classes of mutants might be anticipated from this procedure. This report describes the screening of the Tsg mutants to identify those that survived the enrichment because of a defect in some component required for motility. A group of motility mutants was identified and characterized. Analysis of these mutants and their revertants has indicated relationships among various motile processes.

MATERIALS AND METHODS

D. discoideum Strains

The strains used in these studies were derived from the axenic strain AX3 (10). This strain has been shown to carry mutations at three loci that together confer the ability to grow rapidly in liquid media (14, 19). Isolation of the Tsg mutants has been previously described (6). Those selected for characterization in this study have been designated MC1, MC2, MC3, MC4, MC5, and MC6. They were derived from three independent enrichment procedures: MC1 and MC2 from one; MC3, MC4, and MC5 from another; and MC6 from a third. These strains are defective in motility at restrictive temperature (Mot^{Ts}). The Tsg^+ Mot^+ revertants of MC1 through MC6 chosen for characterization are designated MC11 through MC16, respectively.

Culture Conditions

Cells were grown in HL5 medium (per liter: glucose, 10 g; yeast extract [Difco Laboratories, Detroit, MI], 5 g; proteose peptone [Difco Laboratories], 10 g;

$Na_2HPO_4 \cdot 7H_2O$, 0.35 g; KH_2PO_4, 0.35 g; dihydrostreptomycin sulfate, 50 mg; final pH, 6.4 to 6.6) or in association with *Klebsiella pneumoniae* on SM/5 agar plates (per liter: bactopeptone [Difco Laboratories], 2 g; yeast extract [Difco Laboratories], 0.2 g; glucose, 2 g; $MgSO_4 \cdot 7H_2O$, 1 g; KH_2PO_4, 1.9 g; K_2HPO_4, 0.6 g; agar, 10 g). For experiments requiring large numbers of cells under restrictive temperature conditions, amoebae or spores were plated with bacteria on SM/5 plates, incubated for 24 h at 22°C (permissive temperature) and then shifted to 27°C (restrictive temperature). The number of cells initially plated was chosen so that they would be clearing the bacterial lawn after 16 to 24 h at 27°C. At this time cells were rinsed from the clearing plates with phosphate buffer (50 mM potassium phosphate, pH 6.4), collected by centrifugation (100 *g*, 2 min) at room temperature, and suspended in the same buffer. They were washed essentially free of bacteria by repeated cycles (usually two) of centrifugation. The cell pellet was then suspended as described for each assay. The 27°C incubation did not affect the viability of the mutant strains; their plating efficiencies at 22°C were 0.6 to 0.9 after this incubation. Plaque formation was delayed; plaques appeared after 3 to 7 d rather than 2 to 4.

Cell Migration

The movement of cells across a surface was monitored by plating them on gold-coated cover slips and visualizing the tracks they produced. The gold-coated cover slips were prepared using $HAuCl_4 \cdot 3H_2O$ (Fisher Scientific Co., Pittsburgh, PA), as described (1), except that they were coated with serum albumin the day before the gold was applied rather than the same day. For storage, they were placed in 35-mm petri dishes (Falcon 1008; Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA) in phosphate buffer containing 50 μg/ml streptomycin sulfate and were kept at 4°C for up to 1 wk.

Cells from the *D. discoideum* strain to be tested were stabbed into the center of a bacterial lawn on a nutrient agar plate and incubated at 22°C. For experiments to be conducted at 27°C, the plate was shifted to 27°C 18–24 h before beginning the assay. Cells were collected from the growing rim of the plaque using a wire loop and were dispersed in phosphate buffer or HL5 and counted. They were diluted in HL5 containing 50 μg/ml streptomycin sulfate to a density of 100–1,000 cells/ml, depending on the intended length of incubation on cover slips. The storage buffer was aspirated off the gold-coated cover slips and replaced with 4 ml of the cell suspension. The dishes were then incubated at 22°C or 27°C for 1–24 h, as described for individual experiments. For photography, a cover slip was drained and inverted on a drop of Aqua-Mount (Lerner Laboratories, New Haven, CT). Photographs were taken on Kodak Technical Pan 2415 film at ASA 200 using a Zeiss M35 camera mounted on a Zeiss standard microscope. The microscope was fitted with a ×2.5 objective and a phase 2 condenser with a 15-mm phase ring, which provided dark-field illumination.

Phagocytosis Assay

Cells incubated, collected, and washed as described in the section *Culture Conditions* were suspended in phosphate buffer and incubated with shaking for 1 h at 27°C. They were then concentrated by centrifugation and added to a suspension of *K. pneumoniae* in the same buffer, so that the final concentration of cells was 1 or 2×10^7/ml and the bacteria were at an optical density (550 nm) of 2–3. The mixture was incubated with shaking at 27°C, except where otherwise indicated. At various times, aliquots were withdrawn and added to 4 vol of cold phosphate buffer. The diluted sample was centrifuged at 100 *g* for 2 min to remove the amoebae, and the optical density of the supernatant was measured. The phagocytosis rate (the decrease in optical density with time) was calculated from these measurements by least squares regression analysis. The specific phagocytosis rate is defined as this rate divided by the concentration of *D. discoideum* protein present in the assay mixture.

Oxygen Consumption Assay

Cells incubated, collected, and washed as described in *Culture Conditions* were suspended in phosphate buffer at concentrations between 1 and 6×10^6/ml and shaken at room temperature until assayed, within 1 h 2 ml of cell suspension was sealed into the measurement chamber of a Gilson Oxygraph KM (Gilson Medical Electronics, Inc., Middleton, WI) equipped with a modified Clark electrode, and the suspension was mixed by rapid continuous stirring using a small magnetic stir bar. When a constant oxygen consumption rate (ΔpO_2/min) had been established for 5–15 min, 20 μl of 2 mM dinitrophenol was injected into the chamber, and the resulting oxygen consumption rate was determined. The specific oxygen consumption rate is defined as ΔpO_2/min divided by the concentration of *D. discoideum* protein.

Light Microscopy

For direct examination of cell motility, glass slides were prepared by dipping in a aqueous solution of bovine serum albumin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) followed by absolute ethanol, and then air-dried. Cells were collected from stab plates as described above in the section *Cell Migration*, and a drop of cells in phosphate buffer containing some residual bacteria was applied to the slide. The cover slip was supported on small spots of silicone grease. The cells were examined with a ×100 Zeiss Neofluar objective (phase 3, oil immersion).

Protein Determination

Protein was measured by the Hartree (8) modification of the Lowry assay (11), using bovine serum albumin (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY) as a standard.

RESULTS

Cell Migration

To identify motility-defective strains among the previously isolated Tsg mutants (6), cells were tested for their ability to produce tracks on a gold-covered surface, a technique originally described by Albrecht-Buehler and Goldman (1, 2). Details of the experimental procedure are provided in Materials and Methods. As shown in Fig. 1, amoebae of *D. discoideum* strain AX3 quantitatively remove gold particles from gold-coated cover slips, leaving sharply defined curvilinear records of their passage; the size of the track is a function of the incubation time. This assay provides a convenient screening method that allows many strains to be tested simultaneously for a basic motile activity and requires relatively few cells, many of which can be examined.

The Tsg mutants were tested for track-forming ability after an overnight incubation at 27°C. A numerical score was assigned to each strain based on its average track size, ranging from 0 for no track formation to 5 for the equivalent of the parental control. Of the 126 Tsg mutants tested, 31 were scored 2 or less, indicating that they made tracks in 22 h that were similar to or smaller than 2 h tracks of AX3. Thus all of these strains exhibited significant defects in track formation. 10 strains were particularly defective, reproducibly giving tracks scored 0 or 1. Examples of track patterns and the scores assigned to them may be seen in Fig. 2 and Table I.

16 Tsg mutants that had moderate to severe defects in track formation at 27°C and were close to normal in growth, track formation, and development at 22°C, were chosen for reversion studies. Revertants were selected by plating spores with bacteria at 27°C. Four of the 16 strains failed to yield Tsg^+ revertants at a frequency $>10^{-8}$, and the growth phenotype of two other strains proved to be too leaky to allow the isolation of revertants. Nine strains yielded Tsg^+ revertants at frequencies between 10^{-5} and 10^{-8}, consistent with reversion rates for single mutations. One, MC2, yielded revertants at $\sim 10^{-4}$, despite tight restriction of this mutant. For each of these 10 mutants, up to four (not necessarily independent) revertants were tested for track-forming ability at 27°C. The Tsg^+ revertants of six strains (MC1-MC6) included revertants with improved track-forming ability. For mutants MC1, MC2, MC4, and MC6, all Tsg^+ revertants tested had regained the ability to form tracks, whereas MC3 and MC5 yielded in addition some Tsg^+ revertants that remained defective in track formation. As described later, in the section *Axenic Growth*, MC1 also yielded such revertants under other selective conditions. This class of revertants has not been further characterized.

For each of the mutants MC1 to MC6, one Tsg^+ revertant that had regained track-forming ability was chosen for detailed characterization (strains MC11 to MC16). Tracks made by amoebae of these twelve strains are shown in Fig. 2. As these

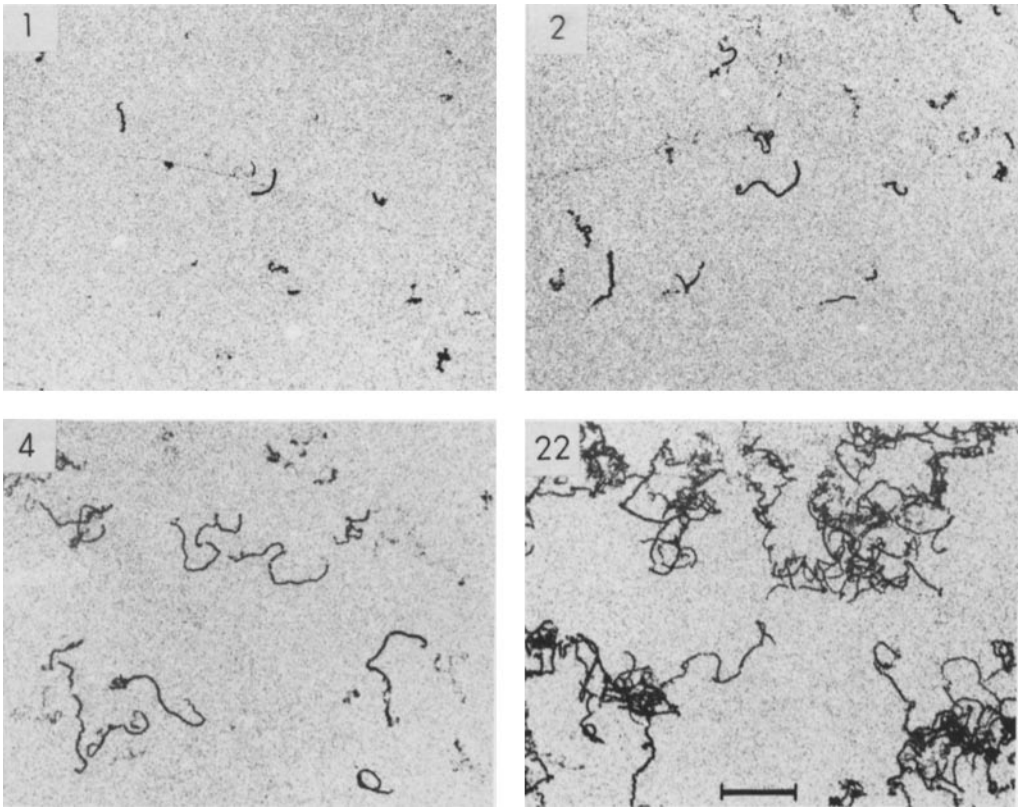

FIGURE 1 Time course of track formation by AX3. Amoebae were plated on gold-coated cover slips after an overnight preincubation at 27°C, as described in Materials and Methods. Incubation was continued at 27°C for the number of hours indicated on each panel. Bar, 0.5 mm. × 20.

patterns illustrate, there is considerable variation in track morphology and degree of defect among the strains. However it is evident that these six Tsg mutants are defective in track-forming ability at 27°C and their Tsg^+ revertants substantially recovered.

Microscopy of Living Cells

The motile behavior of living amoebae was observed with high power phase-constrast optics to determine how the cells' ability to adhere and spread on the substrate, to extend filopodia and pseudopodia, and to translocate, correlated with track-forming ability. None of the six mutant strains exhibited a rapid shut-off of motile functions at 27°C. The time required for evident effect varied from strain to strain, but in all cases several hours were required. After 24 h at 27°C, all of the mutants exhibited severe impairment in migration, spreading, and extension of pseudopodia; by 48 h there was complete loss of motile functions, although the cells remained fairly adherent. In contrast, AX3 amoebae showed at most slight decreases in these activities after 48 h at 27°C. Cells of the revertant strains were substantially more active than those of the corresponding mutants, but were generally somewhat less active than AX3 cells.

Phagocytosis of Bacteria

To determine the ability of these mutants to phagocytose bacteria, a spectrophotometric assay described in Materials and Methods was used. In this assay, uptake of bacteria was directly proportional to the quantity of amoebae in the assay mixture (data not shown), and was a saturable function of bacterial concentration (Fig. 3). A sufficiently high concentration of bacteria was used in the assays to ensure that the uptake rate would be independent of bacterial concentration throughout the assay period. Under these conditions, uptake by AX3 was distinctly biphasic, with a rapid rate for the first 10–12 min, followed by a slower, apparently steady state, rate that was constant for at least 1 h (Fig. 4*a*). Both phases represented uptake and not merely binding, since they were blocked by dinitrophenol, an uncoupler of oxidative phosphorylation (Fig. 4*a*), and by low temperature. The steady state rate was used as a measure of phagocytosis. The kinetics of phagocytosis for a mutant and its revertant are presented in Figure 4*b*. The six mutants MC1 to MC6 proved to be defective in phagocytosis, and their revertants MC11 to MC16 showed significant recovery (Table I). There was good agreement between the extents of the migration and phagocytosis defects for a given strain.

Oxygen Consumption

Rates of oxygen consumption were measured to determine whether the mutants were metabolically active under restrictive conditions. As seen in Table I, only small decreases in oxygen consumption were found; rates for all of the mutants were at least 50% that of the parental control, and there was no pattern of recovery in the revertants. These data do not seem sufficient to account for the much more extreme motility defects; the somewhat lower rates are probably reasonable figures for cells that have not been feeding. Addition of dinitrophenol to 20 μM increased the rate of oxygen consumption by 15% to 50% in AX3 and mutant strains, indicating that the rates reported were coupled to oxidative phosphorylation. Thus by this criterion cell metabolism appears to be normal in these strains at high temperature.

Axenic Growth

All of the experiments described above were carried out using cells grown in association with bacteria. For biochemical

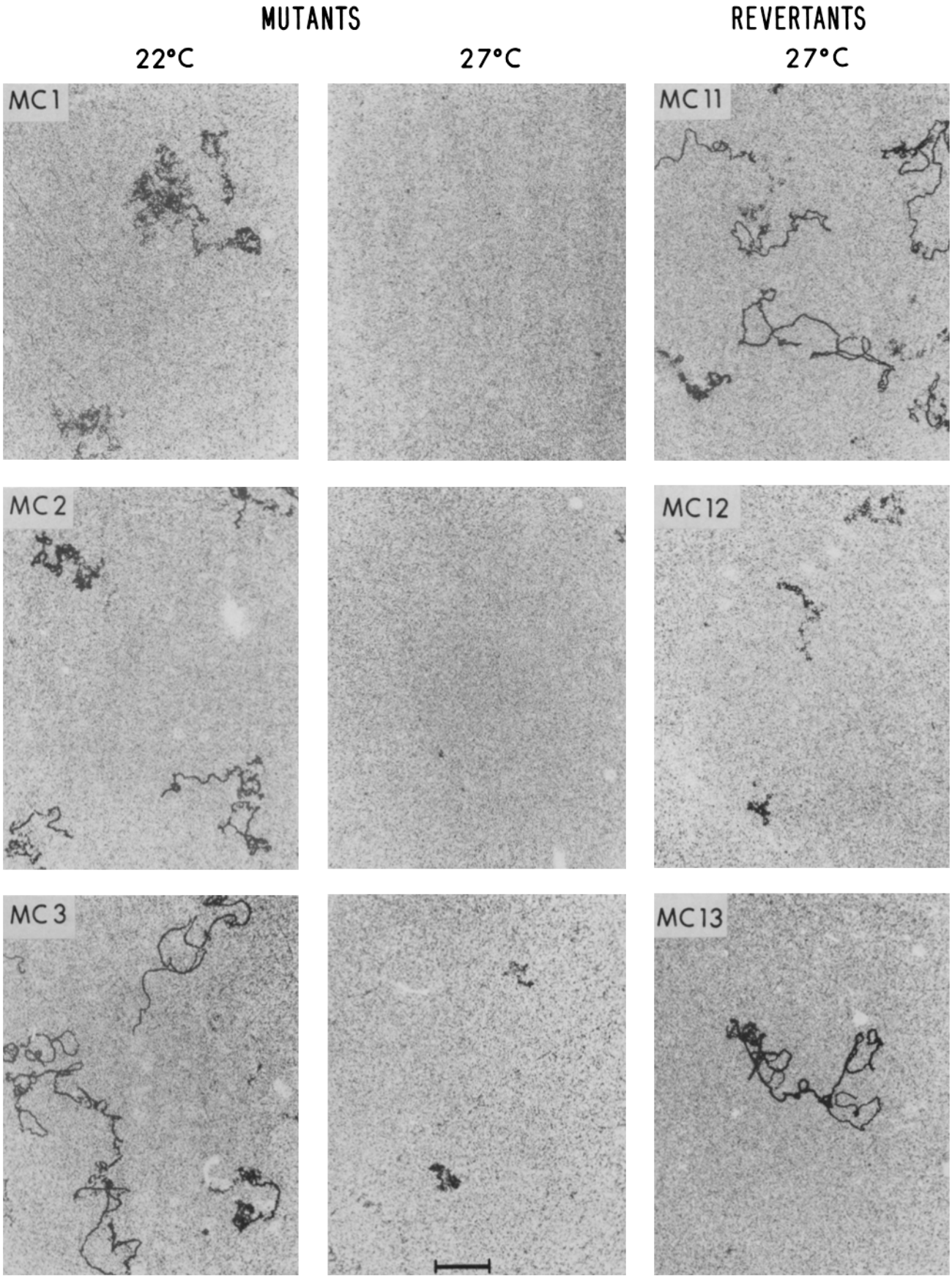

FIGURE 2 Track formation by mutants and revertants. Amoebae were plated on gold-coated cover slips and incubated for 22 h at 22°C or 27°C, as indicated. Cells tested at 27°C had been preincubated at 27°C overnight. Tracks formed by AX3 at 27°C are shown in the last panel of Fig. 1. The motility scores assigned to these strains may be found in Table I. Note the different track morphologies. Bar, 0.5 mm. × 20.

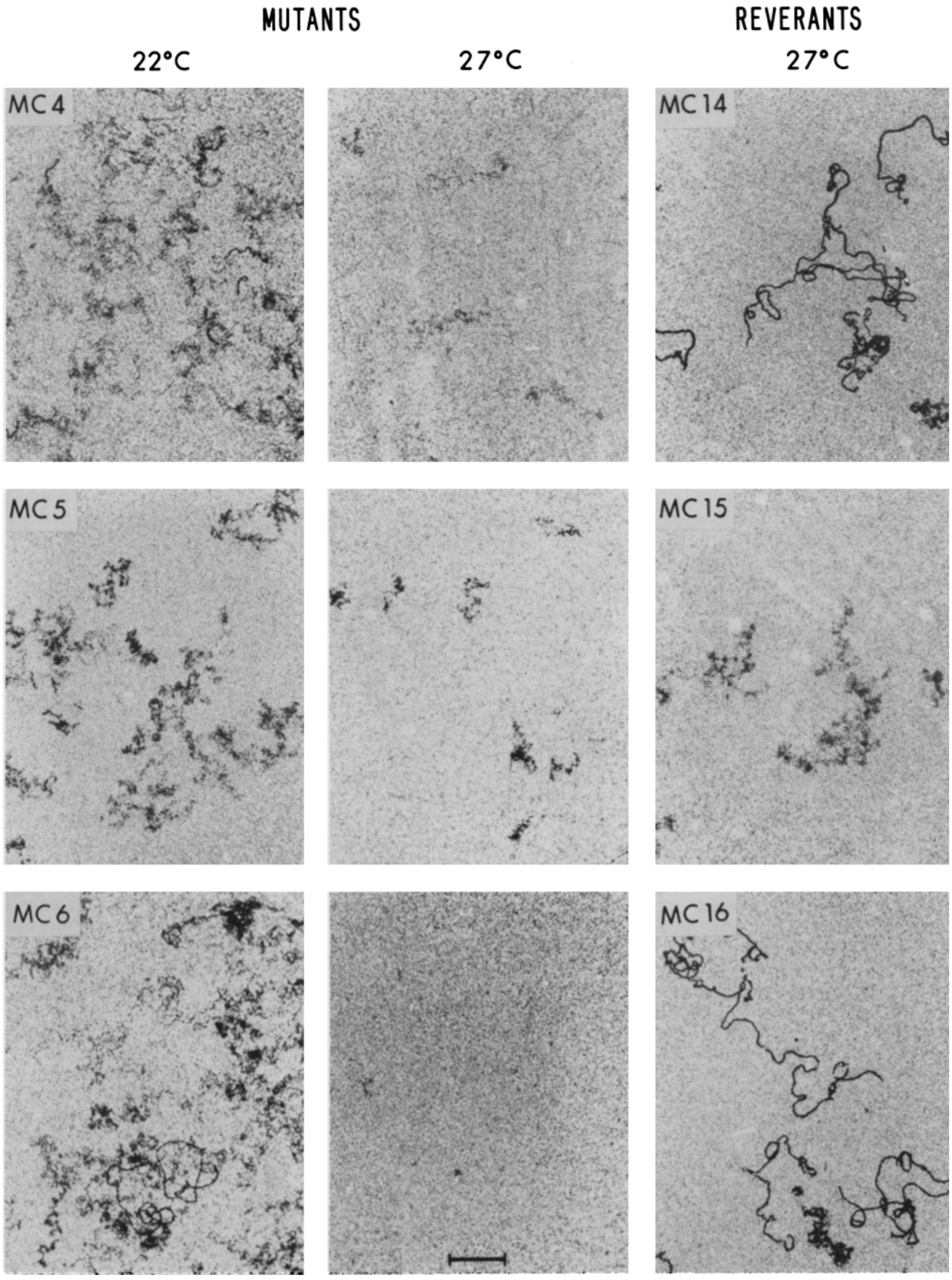

FIGURE 2—continued

characterization, it is more convenient to use cells grown on nutrient media. The parent of the mutants, AX3, is capable of axenic growth, so it was anticipated that the mutant strains would also grow axenically, at least under permissive temperature conditions. However, attempts to establish axenic cultures of the mutants indicated that the axenic growth capabilities of

TABLE I

Properties of Dictyostelium Motility Mutants and Revertants

Strain	Motility on gold	Phagocytosis of bacteria	Consumption of O_2
AX3	5	5.0	5.0
MC1	0	<0.94	5.4
MC11	5	6.5	4.0
MC2	0	<0.84	2.7
MC12	3	1.4	4.0
MC3	1	2.0	4.2
MC13	5	3.6	6.5
MC4	2	<1.4	4.7
MC14	5	4.8	4.0
MC5	2	<1.2	3.4
MC15	4	2.3	7.4
MC6	0	<0.56	2.3
MC16	5	4.0	5.1

All measurements were made after overnight preincubation of the cells at 27°C. The motility scores were assigned on the basis of tracks left by the amoebae during a 22-h incubation on gold-coated cover slips at 27°C (see Fig. 2). Specific phagocytosis and oxygen consumption rates have been scaled, setting the AX3 rate equal to 5, to facilitate comparison with the motility scores. Phagocytosis rates were derived from plots like those shown in Fig. 4. The specific phagocytosis rate for AX3 was Δ0.025 A_{550}·ml/min·mg; all rates were multiplied by 200 to give the numerical values shown in the table. The specific oxygen consumption rate for AX3 was Δ16.7 ± 1.7 mm Hg·ml/min·mg; all rates were multiplied by 0.3 to convert to the figures shown above.

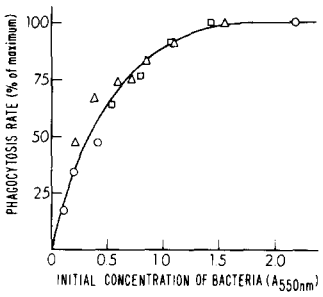

FIGURE 3 Phagocytosis rate as a function of bacterial concentration. The phagocytosis rate of AX3 was determined at 22°C as described in Materials and Methods, except that the assay mixtures contained the indicated concentrations of bacteria. The different symbols represent different experiments.

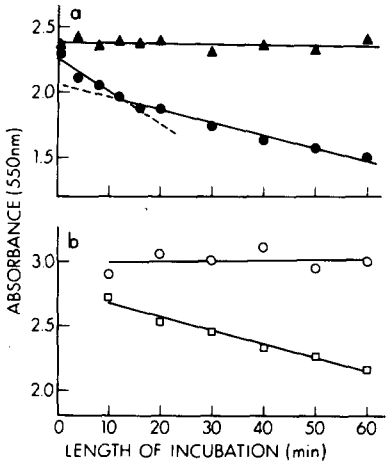

FIGURE 4 Phagocytosis by AX3 and mutant strains. Phagocytosis was measured as described in Materials and Methods. (*a*) AX3 was assayed at 22°C at a concentration of 6.6 × 10^6 cells/ml in the absence (●) and presence of 83 μM dinitrophenol (▲). (*b*) Mutant MC4 (○) and its revertant MC14 (□) were assayed at 27°C at a concentration of 1 × 10^7 cells/ml.

some of these strains had been altered.

The ability of the mutants and revertants to grow on axenic medium under permissive temperature conditions (22°C) is shown in Table II. Many of the strains were slow to establish rapidly growing cultures, as is common for AX3 strains not passaged in axenic culture for many generations. However, two of the mutants, MC1 and MC4, failed to grow axenically even after prolonged incubation, although their revertants MC11 and MC14 grew well. Indeed, MC1 yielded Tsg^+ strains when selected for growth in axenic medium at 22°C; at least one of these was still defective in track formation at high temperature. One of the mutants that did grow axenically, MC2, proved to have a revertant, MC12, that did not. These observations suggest a relationship in some of these strains between the motility mutation and genes affecting axenic growth.

Other studies have shown that nonaxenic *D. discoideum* strains, such as NC4, leave poorly defined, fuzzy tracks on gold-coated cover slips, easily distinguished from the sharp-edged tracks of AX3 (Kayman and Clarke, manuscript in preparation). Some of the mutants also produced this type of track (e.g., MC4 at 22°C). With one apparent exception (MC12), sharp track morphology correlated with an ability to grow axenically. Two of the strains with fuzzy track morphology, MC6 and MC15, were also capable of axenic growth. However when these strains were retested after growth in axenic medium, MC6 gave sharp tracks and MC15 an intermediate pattern. These data also suggest a connection between the two phenotypes.

DISCUSSION

The Tsg mutants examined in this report were isolated by a procedure that enriched for cells unable to incorporate nucleotide from food bacteria into their own DNA at restrictive temperature (6). Strains with temperature-sensitive defects blocking any of a number of processes, including phagocytosis, digestion, and DNA synthesis, might be expected among these mutants. The phagocytosis defective class might itself include strains specifically defective in phagocytosis as well as strains coordinately defective in many or all motile functions. Six members of this latter class, MC1–MC6, were chosen from the

TABLE II

Axenic Growth Capabilities of Mutant Strains

Strain	Generation time*
	h
AX3	10–12
MC1	No growth‖
MC11	10–12
MC2	15–18
MC12	No growth
MC3	10–12
MC13	10–12
MC4	No growth
MC14	10–12
MC5	15–18
MC15	15–18
MC6	15–18§
MC16	15–18

* Growth was measured by counting cells, which were incubated in HL5 medium at 22°C.

‖ Selection for growth in axenic medium yielding Tsg^+ strains.

§ In one experiment, selection for growth in axenic medium yielded a Tsg^+ strain.

large number of Tsg mutants that showed significant migration defects at restrictive temperature in the track formation assay of Albrecht-Buehler (1). These six strains have been designated motility mutants (Mot; in this case temperature-sensitive: Mot^{Ts}) because they were defective at high temperature in many motile functions: spreading, filopodial and pseudopodial activity, migration, and phagocytosis. This constellation of phenotypes appeared not to result from a primary defect in energy metabolism. Furthermore, these phenotypes co-reverted when Tsg^+ revertants were selected.

Most spontaneous Tsg^+ revertants of MC1 through MC6 also recovered phagocytosis, track formation, and other motile activities. The frequencies at which such revertants were isolated are consistent with single genetic events, suggesting that the Ts phenotypes of these mutants resulted from single mutations. That these phenotypes commonly co-reverted is in itself suggestive of single mutations, since the procedure used to isolate revertants did not select for restoration of all the phenotypes. Although plaque formation on bacterial lawns presumably requires phagocytosis, it appears not to require the type, or perhaps degree, of migration activity needed for track formation. This is indicated by the fact that many migration-defective strains yielded Tsg^+ revertants that remained defective in track formation. The genetic separability of these phenotypes also indicates that these two assays measure different motile activities. Taken together, these results suggest that the group of motility defects present in the Mot^{Ts} strains results from the pleiotropic effects of single mutations. This is the implication regardless of the location of the genetic event restoring the Mot^+ phenotype.

There are three lines of evidence suggesting that energy metabolism is not the site of action of the lesions in the Mot^{Ts} strains. First, the Ts phenotypes are reversible: cells held at 27°C until the motility defects were expressed were viable when plated at 22°C, although plaque formation was significantly delayed. Severe blocks in energy metabolism would be expected to result in irreversible damage. Second, the occurrence of revertants whose growth and track formation phenotypes were separated is more readily explained in terms of pseudorevertants of motility-proximal defects than of defects in energy metabolism. Finally, the Mot^{Ts} mutants retained normal, coupled rates of oxidative phosphorylation under conditions in which motile activities were blocked.

Other mutants of *D. discoideum* have been isolated using a different enrichment for phagocytosis defective strains (18). In that study, a class of mutants defective in adhesion and specific types of phagocytosis was chosen for detailed characterization. Motility mutants similar to those described in this report may have been present among their other growth-defective strains, probably grouped in the class they designated IIA. However, this is uncertain because only the growth and phagocytosis properties of that group of mutants were examined.

The results of our study suggest that the cellular mechanisms responsible for migration, phagocytosis, and axenic growth share common elements. Reversion data with respect to axenic growth for four of the six motility mutants (MC1, MC2, MC4, and MC6) indicate a possible relationship between their mutations and axenic growth. Other studies, comparing pinocytosis in the axenic strain AX3 and the nonaxenic strain NC4, have shown that the nonaxenic strain exhibits a much lower rate of pinocytosis (Kayman and Clarke, manuscript in preparation). At least some of the variation in axenic growth among the mutant and revertant strains is also associated with differences in pinocytosis rates (data not shown). An additional correlation was found between track morphology and axenic growth capacity in these strains, adding to other evidence (Kayman and Clarke, manuscript in preparation) that the mechanisms of track formation and axenic growth are related. The present results also demonstrate that the two phenotypes can be separated. The relationships among these different motile activities are currently being explored by further biochemical and genetic studies of the mutants, including complementation analysis. Preliminary data suggest that the Mot^{Ts} mutations in MC1 to MC6 represent a minimum of five complementation groups.

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