GENETIC AND MORPHOLOGICAL STUDY OF AGGREGATION IN THE CELLULAR SLIME MOLD *POLYSPHONDYLIUM VIOLACEUM*[1,2]

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ABSTRACT

A system for genetic analysis in the cellular slime mold *P. violaceum* has been developed. Two growth-temperature-sensitive mutants were isolated in a haploid strain and used to select rare diploid heterozygotes arising by spontaneous fusion of the haploid cells. A recessive mutation to cycloheximide resistance in one strain enables selection of segregants, which often appear to be aneuploid.—Aggregation-defective (ag⁻) mutants having a wide range of phenotypes were isolated in both temperature-sensitive strains after nitrosoguanidine treatment, and complementation tests were performed between pairs of these mutants. Of 380 diploids isolated, 32 showed defective aggregation and were considered to contain 2 noncomplementing ag⁻ mutations. Among noncomplementing mutants interallelic complementation is common. Noncomplementing mutants fall into 4 complementation groups, and those within each complementation group are phenotypically similar. Statistical analysis of the results suggests that the number of complementation units involved in aggregation is about 50.

IN the life cycle of cellular slime molds such as *Dictyostelium discoideum* and *Polysphondylium violaceum*, growth is separated from morphogenesis and differentiation (BONNER 1967). During the vegetative phase, autonomous amebae consume bacteria, grow, and divide. Upon depletion of the food supply, the amebae enter interphase, a period of several hours during which they differentiate into aggregation-competent cells. Scattered amebae then emit signals, and other amebae begin to stream together toward them to form multicellular pseudoplasmodia that develop into fruiting bodies (Figure 1). BONNER (1947) showed that aggregation is a chemotactic event; amebae move toward a higher concentration of a substance called acrasin produced by other amebae. Cyclic-AMP (cAMP) has been identified as the acrasin for *D. discoideum* (KONIJN, *et al.* 1967). Several other species of Dictyostelium respond chemotactically to cAMP, but *P. violaceum* does not, and its acrasin is not yet known (BONNER *et al.* 1972).

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FIGURE 1.—Life cycle of *Polysphondylium violaceum*. Upon depletion of the food supply, amebae stream together to form an elongated mass of cells or pseudoplasmodium. This cell mass develops into a fruiting body composed of stalk cells and spores, which germinate into amebae.

The process of aggregation in cellular slime molds involves intercellular communication, morphogenetic movement, and cellular adhesion, phenomena essential to developmental processes in higher animals. Since aggregation occurs after growth and among free cells, it is more easily studied than most morphogenetic events; and, since slime mold cells are normally haploid, mutants defective in this process can be isolated readily.

The work reported here was directed toward estimating the number of genes whose products are involved in aggregation in *P. violaceum*. Our approach was analogous to that used by BENZER (1961) to estimate the size of the *r*II cistrons of bacteriophage T4. Many aggregation-defective (ag^-) mutants were isolated, and complementation tests were performed between pairs of these using a method for diploid selection developed in *D. discoideum* (LOOMIS 1969; KATZ and SUSSMAN 1972). Enough mutants were isolated so that noncomplementing pairs were found and a statistical estimate of the number of genes controlling aggregation could be made (WARREN, WARREN and COX 1975). In this paper we present a detailed description of the genetic system, complementation results, phenotypes of the aggregation defective mutants, and statistical methods.

MATERIALS AND METHODS

Organisms: *Polysphondylium violaceum* strain No. 1, kindly supplied by J. T. BONNER, was grown on *Escherichia coli* B/r on agar plates.

Nutrient media: Nutrient agar (NA) (BONNER 1947). GYP medium (WEBER 1970). GYP plates contain GYP medium solidified with 2.5% agar. Soft agar is GYP medium containing 0.8% agar.

Assaying and cloning cells: Amebae were suspended in 1% Bonner's standard solution (BSS) (BONNER 1947) and spores in Vogel and Bonner buffer (VOGEL and BONNER 1956). One drop of *E. coli* B/r (henceforth referred to as B/r) and 0.1 ml of cell suspension (2×10^3/ml) were added to 2.5 ml soft agar at 38–41° and immediately poured onto a 15 × 100 mm GYP agar plate. Upon incubation at 21°, plaques became visible in the bacterial lawn in about 2 days and were easily counted after 3.5 days. At 30° plaques became visible in about 1.5 days. Clones were isolated from plates containing only about 50 plaques. Single plaques were cut out and spread on 15 × 60 mm GYP agar plates with B/r.

Mutant isolation: Nitrosoguanidine mutagenesis: The method was similar to that of YANAGISAWA, LOOMIS and SUSSMAN (1967). Amebae were suspended in 0.05 M potassium phosphate buffer, pH 6.0, at a concentration of 2.0×10^7 per ml. Ten ml of cells were added to 10 ml of a

N-methyl-N'-nitro-N-nitrosoguanidine (NG) solution (2 mg/ml in the same buffer) and incubated in a flask at 22° with shaking for 45 min. The cells were chilled, washed twice in phosphate buffer, and spread with B/r on GYP plates. When amebae had grown up, they were removed from the growth plates in BSS and cloned. All mutants derived from different growth plates are independent. Survival after NG treatment was determined by plating diluted untreated cells and various concentrations of treated cells on GYP with B/r. Survival varied from 0.4% to 6%.

Isolation of temperature sensitive (Ts) mutants: A sterile toothpick was jabbed into a plaque and then into 2 GYP plates seeded with bacteria in an agar overlay. The plate jabbed first was incubated at 30°, the second at 21°. About 50–60 clones could be tested per plate. After 2 days at 30° and 3 days at 21°, any clone that showed a clear plaque at 21°, but not at 30°, was retested. From 4582 NG treated clones tested, 6 clean Ts mutants were isolated. Each gave no plaques when 10^6 cells were plated at 30°. Two mutants, *ts119* and *ts152*, which grew well at 21° and developed normally at 21° and 30° were chosen to use for diploid selection.

Isolation of a cycloheximide resistant (cy^R) mutant: Spores from NG treated *ts119* cells were plated with B/r in an agar overlay on GYP plates containing 0.3 mg/ml cycloheximide (CY-GYP plates). Plaques became visible after 3.5 days at 21° at a frequency of 6×10^{-6}. One was chosen for use; its efficiency of plating on CY-GYP is 0.2–0.6 that on GYP, and plaques do not appear on CY-GYP plates until 3–4 days compared to 2 days on GYP plates.

Isolation of morphologically abnormal mutants: Toothpicks were jabbed into plaques from NG treated *ts119*cy^R and *ts152* cells and then into GYP plates seeded with bacteria in an agar overlay. After 3–4 days at 21°, most clones on a plate would usually have developed fruiting bodies. Any clones in which the cells did not show signs of streaming or did not form tight lumps of cells were retested as possible aggregation-defective (ag⁻) mutants. Other very obvious morphologically abnormal mutants were also isolated. Plaques were retested several times and then cloned. After NG treatment with 0.5% survival, 24 ag⁻ and 29 other morphological mutants were isolated from 6599 *ts152* clones tested. With *ts119*cy^R cells, 2 separate NG treatments were done. In treatment A, survival was 6%, and 20 ag⁻ and 26 other morphological mutants were isolated from 6152 clones tested. In B, survival was 0.4%, and 31 ag⁻ and 51 other mutants were isolated from 2796 clones. All mutants derived from different growth plates are independent; those from the same growth plates have a very low probability (less than 10^{-5}) of being the same. All mutants in the same complementation group, with the exception of *ts152–938*, are known to be derived from different growth plates. The origin of *ts152–938* is unknown; however, this mutant is phenotypically distinguishable from all other *ts152* mutants determined to be in the same complementation group and thus can be considered independent of them.

Complementation tests. Mixing mutants: *Ts119*cy^R ag⁻ mutants and *ts152* ag⁻ mutants were grown together in pairs so that some would fuse to form diploids. However, since some ag⁻ mutants form fruiting bodies when mixed together (in the absence of fusion), pairs of *ts119*cy^R ag⁻ mutants and pairs of *ts152* ag⁻ mutants were also grown together to test for synergism. Three drops of B/r and 0.1 ml of each of 2 mutants at 2×10^6/ml were spread on an NA plate and incubated at 21°. These plates were examined after 3 and 5 days incubation. Wild-type (ag⁺) cells cover such plates with fruiting bodies by 3 days.

Isolation and characterization of diploids: After incubation for 6 days at 21°, cells were harvested from *ts119*cy^R × *ts152* mix plates, and about 10^7 cells were plated with B/r in an agar overlay on each of several GYP plates. These were incubated at 30°. After 2–4 days plaques became visible; usually such temperature-resistant (Tr) clones appeared at a frequency of 10^{-6} — 10^{-7}, but sometimes they were very rare. Tr plaques were cut out and spread on 15 × 60 mm GYP plates with bacteria. These plates were incubated at 30° and observed several times to determine their aggregation phenotypes. Ploidy of the Tr clones was first checked by spore size (SUSSMAN and SUSSMAN 1962) in all cases where spores formed. In addition, all ag⁻ diploids were cloned and checked to show that they were cycloheximide-sensitive, that they segregated cycloheximide-resistant cells, and that some of the cycloheximide-resistant cells were temperature-sensitive. Twenty cycloheximide-resistant segregants from each of 5 clones of each ag⁻ diploid were tested for growth at 21° and 30°. Aggregation phenotypes of some of the

cycloheximide-resistant segregant clones were determined by spreading plaques on 15 × 60 mm GYP plates with B/r.

Determination of generation times: Amebae were grown on NA plates, harvested in BSS, and diluted to 10^6 per ml. Then 0.1 ml of each strain used was spread with B/r on each of 10 NA plates and incubated at 21°. After 10 and 28 hours, the number of cells on each of 5 plates was assayed. Generation times were calculated from the ratio of the average number of cells at 28 hours compared to 10 hours. Experiments in which cells were assayed at many times showed exponential growth from 4 to 32 hours, the last time point used.

Growing cells for photography: For Figures 3–8 and 10–13 conditions were controlled so that photographs taken at each of several times would be directly comparable. About 10^5 amebae were spread with B/r on each of several 15 × 60 mm plates containing 10 ml GYP agar. Plates were incubated at 21°. After 24 hours, cells from one plate in each set were counted in a hemacytometer. Only if the number of cells was close to 10^7, which is 2 divisions (6–7 hours) away from the final possible number of cells per plate, would the rest of the plates in the set be photographed at later times. On GYP plates, a square 2 mm on a side encloses about 5×10^4 cells (2 mm markers are in the Figures).

RESULTS

Genetic system: In order to do complementation tests with aggregation defective (ag^-) mutants, diploids formed from ag^- haploids must be isolated. LOOMIS (1969) and KATZ and SUSSMAN (1972) developed a method in *D. discoideum* for selecting rare diploids resulting from the spontaneous fusion of two genetically different haploid cells. The diploid cells go through the same developmental stages as the haploid ones.

The same method for diploid selection was used here. Two growth temperature-sensitive (Ts) mutants (*ts119* and *ts152*) were isolated after nitrosoguanidine treatment; they grow at 21°, but not at 30°. After being grown together at 21° for 6 days, one in 10^6 to 10^7 cells will grow when plated at 30°, each cell forming a clear plaque in the bacterial lawn. If a plaque is cut out and spread on an agar plate with bacteria, at 30° the cells grow and form fruiting bodies covering the surface. Some such clones have small ellipsoidal spores and are probably temperature-resistant revertants of *ts119* and *ts152* or, possibly, haploid segregants of diploids. Most clones, however, especially those arising from the biggest plaques, have spores characteristic of diploid cells (SUSSMAN and SUSSMAN 1962). They are about 1.5 times as long as haploid spores and often have abnormal banana-like shapes. Haploid and diploid spores can be distinguished readily, as may be seen in Figure 2. The large-spored clones are diploid heterozygotes in which the temperature sensitive mutations complement each other; that is, they are *ts119/ts119+*, *ts152/ts152+*.

Haploid segregants were selected from diploid clones by the method employed by KATZ and SUSSMAN (1972). A mutation to cycloheximide resistance (cy^R) was selected in one of the temperature-sensitive strains (*ts119*). Diploids formed by fusion of *ts119*cy^R and *ts152* cells are phenotypically cycloheximide-sensitive and genotypically heterozygous (cy^R/cy^S). Since sensitivity is dominant, hemizygous cells containing the chromosome carrying the cy^R mutation, but lacking its cy^S homolog, can be selected by plating large numbers of diploid cells on cycloheximide medium. These cells are found at a frequency of about 10^{-3} to 10^{-4}

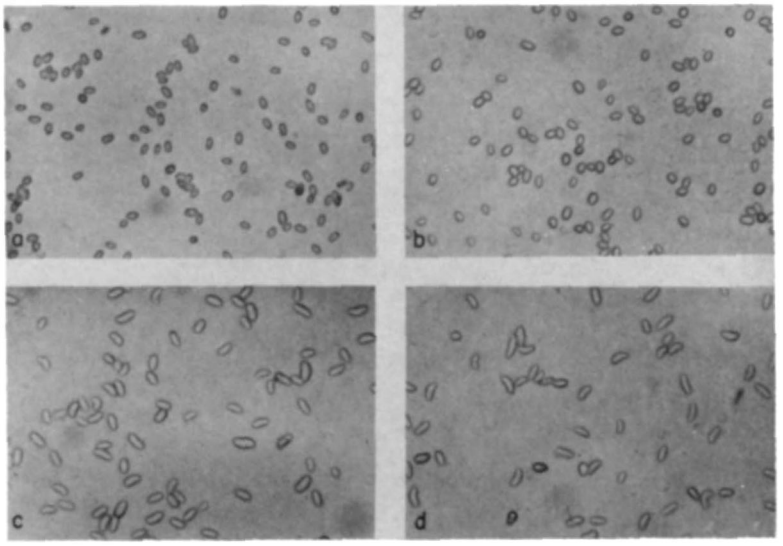


FIGURE 2.—Spores of *P. violaceum*. (a) Haploid (wild type); (b) haploid (*ts152*); (c and d) diploid.

in a diploid population grown from a plaque to 10^7 cells, whereas haploid cy^S cells will give no clearing of the bacterial lawn even when 10^8 cells are plated on cycloheximide.

Even though the segregants selected on cycloheximide medium usually have small spores, they are often probably not haploid but aneuploid, since cycloheximide resistant segregants of *ts119*/+, cy^R/cy^S, *ts152*/+ diploids sometimes have abnormal phenotypes. There are 6–8 commonly observed types, and most of them have been cloned and shown to segregate normal as well as abnormal types. Two have been extensively cloned and recloned; one of these makes fruiting bodies with very thick stalks and reduced sori, and the other forms sparsely scattered lumps, slugs, and kinky stalks. Upon cloning, each of these gave rise to normal types and abnormal ones similar to the original isolates, but showing varying degrees of abnormality. That is, different numbers of normal fruiting bodies were scattered among the abnormal structures. Upon recloning, the normal ones gave rise to all normal clones, while the abnormal ones gave rise to both normal and abnormal ones. The more abnormal types gave rise to a smaller fraction of normal clones than did those that had some scattered good fruiting bodies. These results are consistent with the expected behavior of aneuploid clones that become normal upon complete haploidization. Proof that they actually are aneuploids will require chromosome counts. Since spores are usually small, these putative aneuploids must not have many extra chromosomes.

Phenotypes of aggregation defective (ag⁻) mutants: Ag^- mutants in both temperature-sensitive strains vary in their behavior, as Figure 3 shows. A few seem to do nothing after growth; the cells are fairly evenly spread over the agar surface. Many of the mutants form loose mounds of cells that give a somewhat

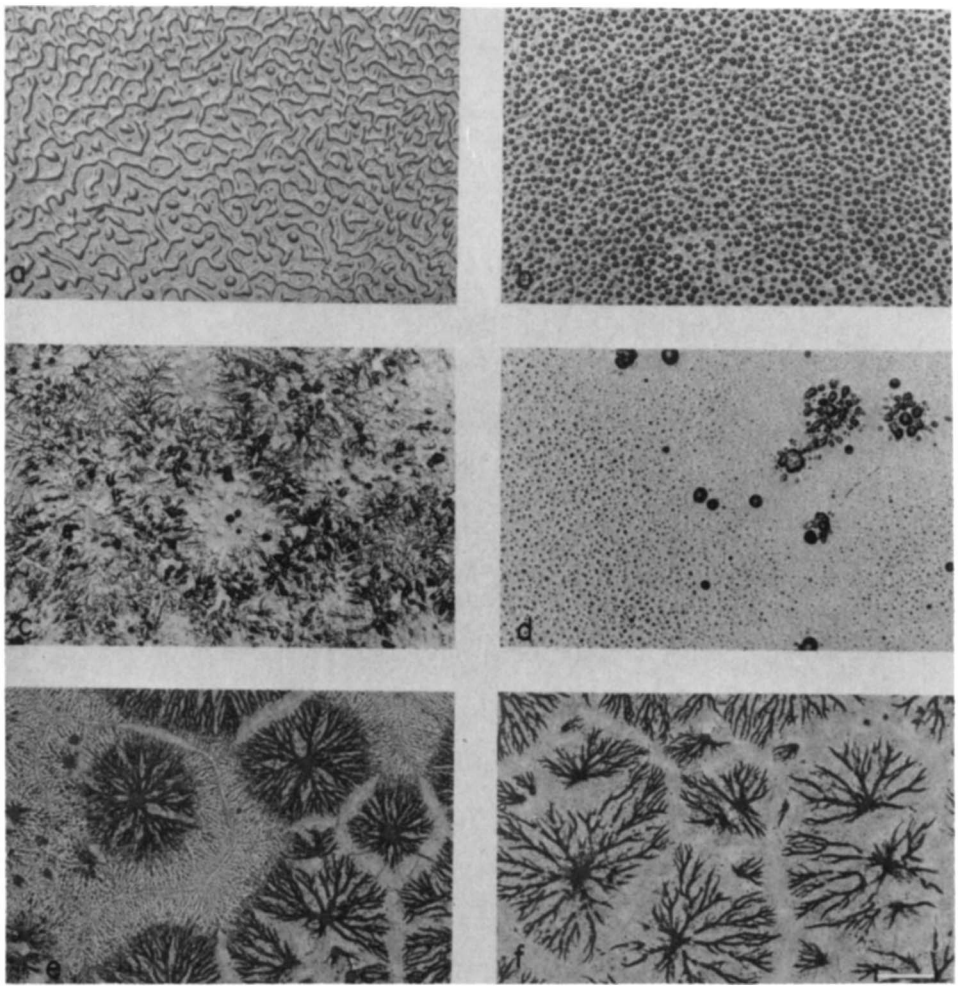


FIGURE 3.—Cultures of ag⁻ and ag+ cells. (a–d) Four *ts119cy*^{R} ag⁻ mutants after incubation for 50 hours at 21° [(a) *B349*, (b) *B344*, (c) *A310*, (d) *B416*]; (e) ag+ cells after 40 hours; (f) ag+ cells after 43 hours. Bar represents 2 mm. All cultures standardized as described in MATERIALS AND METHODS.

pebbled appearance to the plate, although no streaming is seen. These mounds can be rounded up to varying extents (Figure 3, a and b), but are never like the tight lumps resulting from normal aggregation (Figure 3, e and f). Some mutants manage to form streams of cells (Figure 3c), but these look unorganized and do not result in the formation of tight lumps. In most cases the streams gradually disappear long before the cells die (Figure 4). Many of the ag⁻ mutants eventually form a few minute fruiting bodies, and some will form many if left long enough.

It is possible that some of the ag⁻ mutants have metabolic defects not directly related to aggregation that, nonetheless, result in loss of the ability to aggregate.

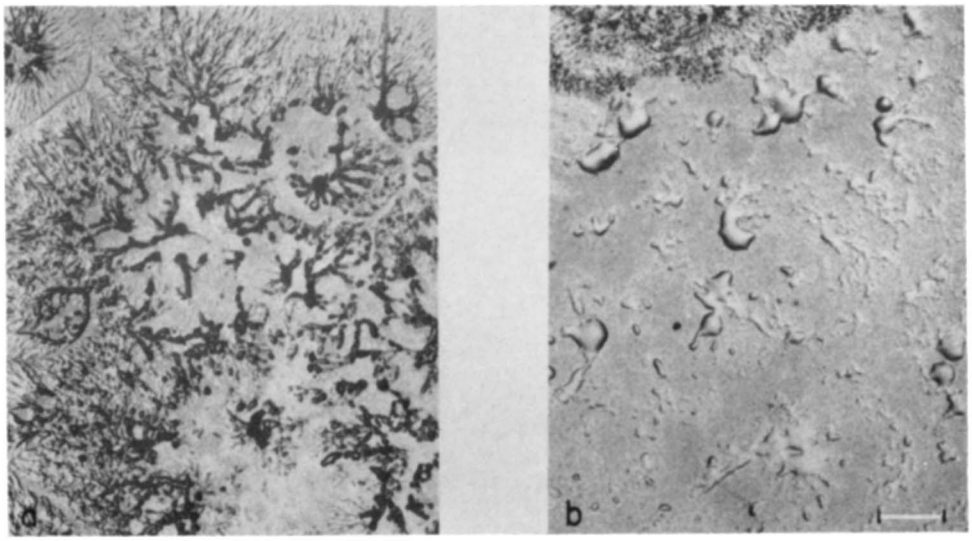


FIGURE 4.—An ag⁻ mutant, *ts119cy^R-B8*, after 50 hours (a) and 75 hours (b) incubation at 21°. At the later time most of the streams have disappeared and the cells are in loose lumps. Bar represents 2 mm. Cultures standardized as described in MATERIALS AND METHODS.

In an attempt to rule out this possibility, the generation times of many mutants were determined. As Table 1 shows, most of them grow as well as ag⁺ cells of the corresponding temperature-sensitive strain and, therefore, probably do not have general metabolic defects. A few have generation times 10–20% longer than corresponding ag⁺ cells, and this small decrease in growth rate might possibly account for the ag⁻ phenotype.

Whereas ag⁻ mutants show a wide variety of phenotypes, those within a complementation group (CG) resemble each other. As will be shown later, four complementation groups have been defined. Mutants in CG I, shown in Figure 5, all form loose mounds of cells, some of which are more rounded than others. These mutants are all leaky; that is, they will eventually form fruiting bodies under some conditions. Two of them never form fruiting bodies under normal conditions, but may if there is yeast contamination on the plate. Three of them form widely scattered, tiny, unbranched fruiting bodies several days after growth is completed. The other two, *ts119cy^R-B515* and *ts152–335*, will form many fruiting bodies upon prolonged incubation, as Figure 6 shows. Although *ts152–335* cells eventually cover the plate with tiny fruiting bodies, aggregation is definitely abnormal as well as late, and there is a large background of unaggregated cells. Figures 7 and 8 show mutants in complementation groups III and IV; again, alleles in each group resemble each other. Mutants in CG II have the same range of phenotypes as those in CG I.

In addition to a shared morphology, all mutants in CG I have an identifiable phenotype; they promote aggregation when mixed with any mutant outside CG I,

TABLE 1

Comparison of generation times in ag+ *and* ag- *cells*

Strains	Generation* times (hours)	Average gen. time (hours)	% difference in gen. time from *ag*+
ts119cy^{R}			
ag+	3.6(15%), 3.6(19%),† 3.9(10%), 3.5(14%)	3.7	—
A310	3.9(17%)	3.9	+5
A577(II)‡	3.7(10%)	3.7	0
A657	3.8(11%)	3.8	+3
A1142(II)	3.8(15%)	3.8	+3
A1231(II)	3.8(17%)	3.8	+3
B202	3.7(16%)	3.7	0
B307	4.1(13%)	4.1	+11
B344	3.6(8%)	3.6	—3
B349(I)	3.5(14%), 3.8(20%),	3.7	0
B586(I)	3.5(16%)	3.5	—5
ts152			
ag+	3.1(13%), 3.2(13%), 3.1(12%), 3.1(20%)	3.1	—
183(II)	3.2(8%), 3.0(16%)	3.1	0
188	3.1(15%)	3.1	0
279	3.6(10%)	3.6	+16
295(II)	3.1(14%), 3.0(12%)	3.1	0
547(III)	3.2(13%)	3.2	+3
561	3.8(14%), 3.5(10%)	3.7	+19
678(I)	3.3(19%), 2.9(16%)	3.1	0
788(II)	3.3(11%)	3.3	+6
903(III)	3.3(16%)	3.3	+6
936(I)	3.1(8%)	3.1	0
938(II)	3.1(16%)	3.1	0

* Generation times were determined by comparing the average number of cells on 5 growth plates incubated 10 hours at 21° to that on 5 growth plates incubated 28 hours.

† Numbers in parentheses are percentage error; they are the sum of the standard deviation of the average number of cells on plates incubated 10 hours expressed as a percentage and the corresponding percentage from 28 hour plates. Results with percent error greater than 20% are not shown.

‡ Roman numerals designate complementation groups.

but not when mixed with each other, as is shown in Figure 9. Usually pairs of ag- mutants grown together look like one or the other or a combination of both types, but mixtures of CG I mutants with other ag- mutants form fruiting bodies almost as soon as wild type controls. The fruiting bodies vary in morphology depending on which mutants are used (Fig. 9). All mutants that promote this synergistic development are in CG I.

There are mutants not in CG's I, II, or IV that look similar to mutants assigned to these groups; there are also other cases of mutants that look similar to each other but complement. All of the mutants we have studied can be grouped

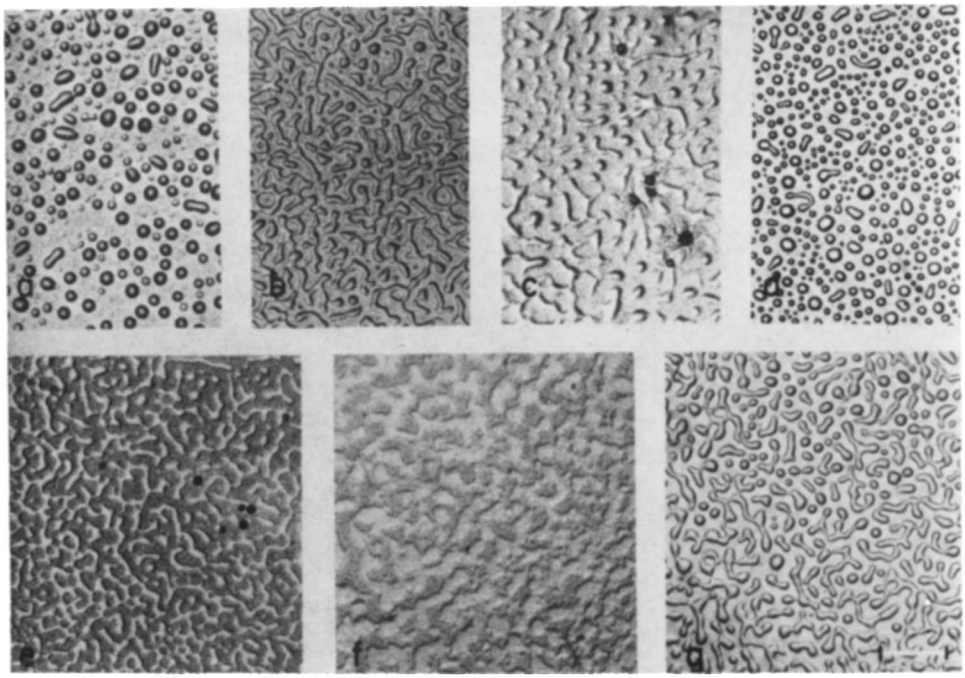


FIGURE 5.—Mutants in complementation group I after 50 hours incubation at 21° (a–d) *ts119cy*^{R} mutants; (a) *B4*, (b) *B349*, (c) *B515*, (d) *B586*; (e–g) *ts152* mutants; (e) *335*, (f) *678*, (g) *936*. Bar represents 2 mm. Cultures standardized as described in MATERIALS AND METHODS.

roughly into about 12 phenotypic classes, and in general a phenotypic class can represent more than one complementation group, while to date mutants within a complementation group represent only one phenotypic class.

Complementation tests: Each complementation test was performed as described in the MATERIALS AND METHODS section. In many cases, ag^{-} diploids containing 2 noncomplementing ag^{-} mutations) develop better than either parental type, and some eventually make fruiting bodies sparsely distributed over the whole agar surface. All, however, are clearly distinguishable, morphologically and temporally, from wild-type diploids and those containing complementing ag^{-} mutations.

To illustrate these general results, the following contrasts development in two ag^{+} diploids with an ag^{-} diploid. The ag^{-} diploid chosen is one of the very "leaky" ones; that is, it makes more fruiting bodies than most others. Figure 10 shows that aggregation in a wild-type diploid, *ts119cy*^{R} *ag*$^{+}$, *ts152ag*$^{+}$, and in a diploid containing 2 complementing ag^{-} mutations, *ts119cy*^{R}*-A205,ts152–936*, is quite comparable, even at early stages when some bacteria remain at the edge of the plates. Figures 11 and 12 compare development at later times in these ag^{+} diploids (+,+ and *A205,936*) with a diploid containing 2 noncomplementing mutations, (*ts119cy*^{R}*-B515, ts152–678*). The noncomplementing diploid is leaky, but is easily distinguished from diploids showing normal aggregation. Even at 50

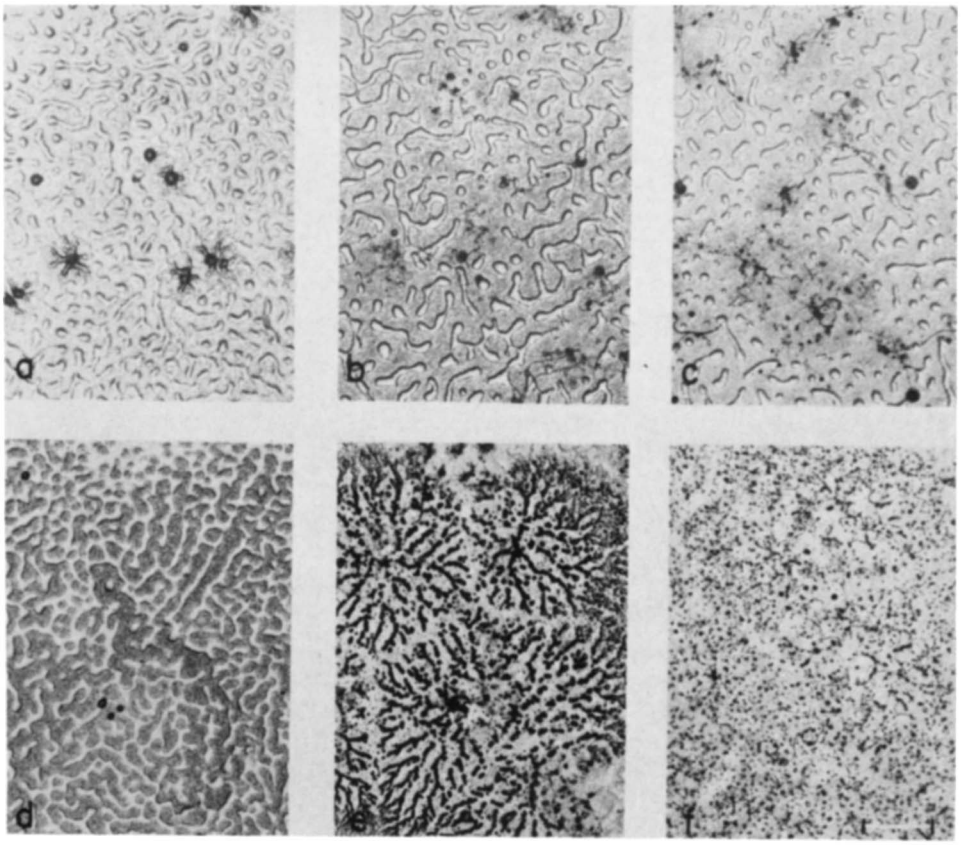


FIGURE 6.—Development of two complementation group I mutants upon prolonged incubation. *Ts119cy^{R}-B515* cultures after (a) 50, (b) 75, and (c) 100 hours incubation at 21°; *ts152–335* cultures after (d) 50, (e) 75, and (f) 100 hours. Bar represents 2 mm. Cultures standardized as described in MATERIALS AND METHODS.

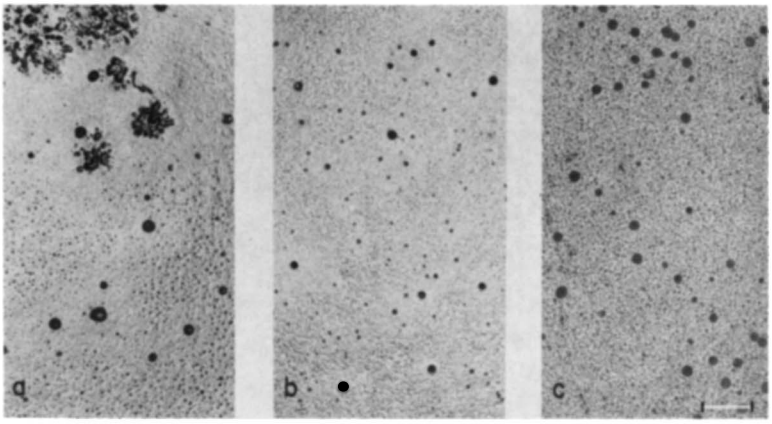


FIGURE 7.—Mutants in complementation group III after 50 hours incubation at 21° (a) *ts119cy^{R}-B416*, (b) *ts152–903*, (c) *ts152–547*. Bar represents 2 mm. Cultures standardized as described in MATERIALS AND METHODS.

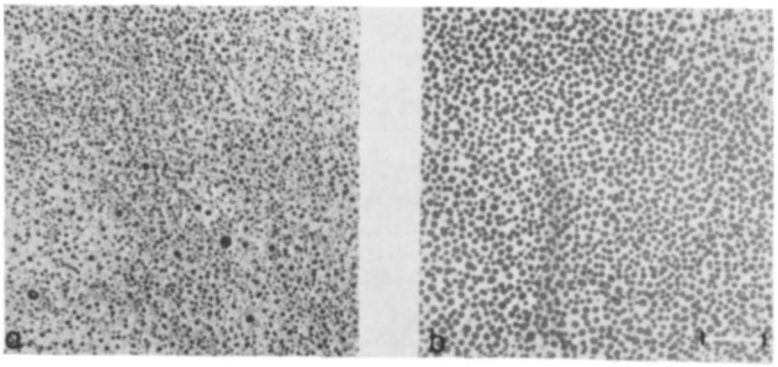
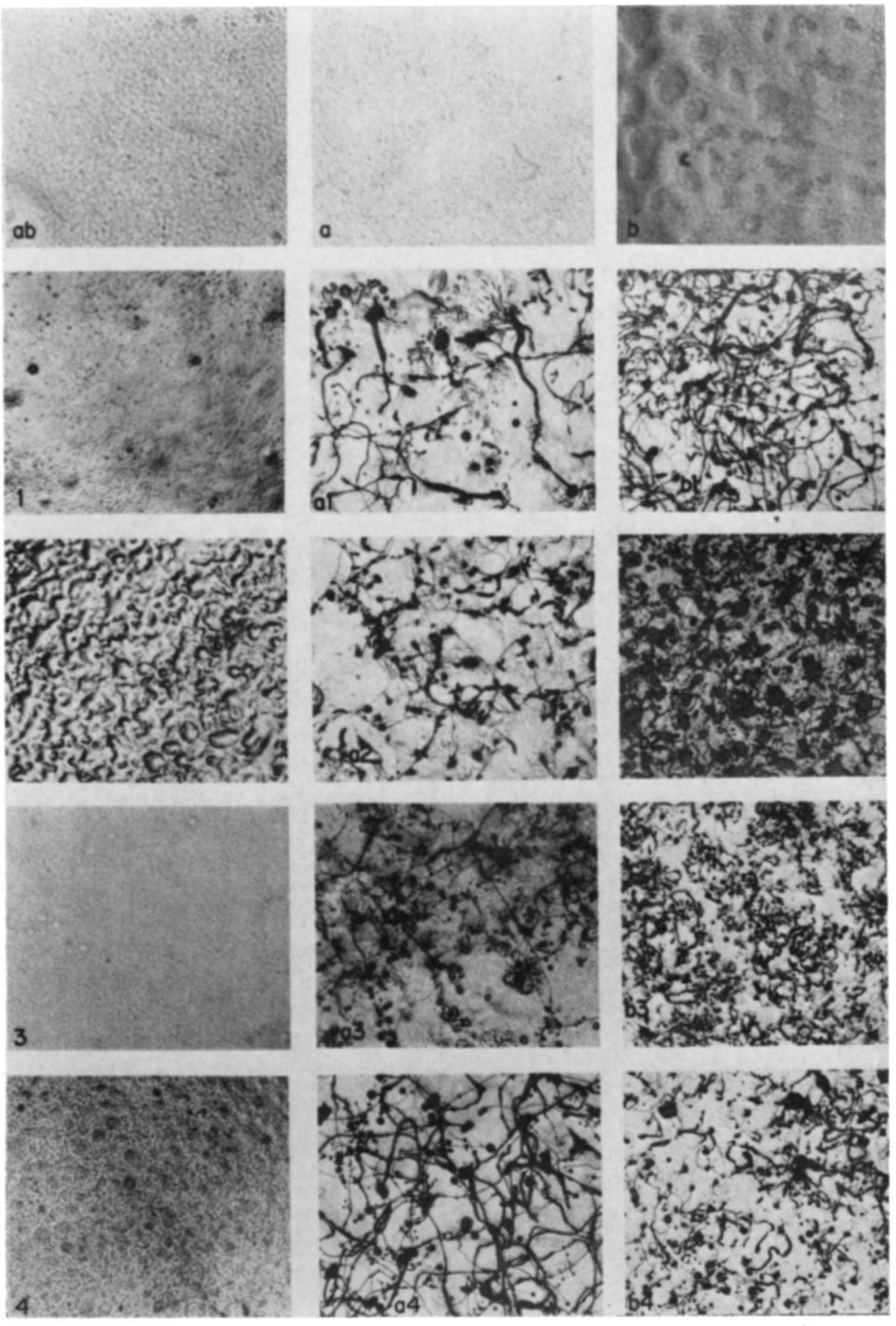


FIGURE 8.—Mutants in complementation group IV after 50 hours incubation at 21°. (a) *ts119cy^R–A852*, (b) *ts152–478*. Bar represents 2 mm. Cultures standardized as described in MATERIALS AND METHODS.

hours, when the ag$^+$ diploids have formed early fruiting bodies, most of the *B515,678* cells are still in loose mounds, and only a few tiny lumps have formed (Figure 12). As Figure 13 shows, *B515,678* eventually starts to aggregate and later forms tiny fruiting bodies over the whole plate. The parental haploids are both less leaky than this diploid, but one of them, *ts119cy^R-B515*, does form some aggregates and fruiting bodies after prolonged incubation (see Figure 6). The development of this diploid very closely resembles that of a third mutant in the same complementation group, *ts152–335* (also Figure 6).

Figure 14 shows the results of the complementation tests completed so far. Using 17 *ts152* ag$^-$ mutants and 24 *ts119cy^R* ag$^-$ mutants, 380 of 408 possible diploids have been isolated. Thirty-two of them contain noncomplementing ag$^-$ mutations. In these experiments, two ag$^-$ mutations were considered to complement if aggregation was as normal in the diploid containing both of them as in diploids formed by fusion of each ag$^-$ mutant with an ag$^+$ cell of the other temperature-sensitive strain; otherwise, they were considered to be in the same complementation group. Of the 41 ag$^-$ mutations studied, only one was found to have any effect when present with the wild-type allele in a heterozygote. Diploids containing *A559*, including *ts119cy^R–A559*, *ts152–ag$^+$*, aggregated somewhat more slowly than normal and formed tiny shrubby fruiting bodies. All other ag$^-$/ag$^+$ diploids developed normally.

The patterns of complementation and noncomplementation in the matrix in Figure 14 are consistent. Mutants that do not complement a given mutant in the other strain show identical patterns of complementation and noncomplementation with all other mutants in that strain. Thus, there is no evidence for double mutants. Noncomplementing mutants fall into 4 complementation groups. Mutants were isolated in 2 of them with high frequency; of the 41 mutants studied, 8 are defective in CG I and 8 in CG II, while there are 5 altogether in CG's III and IV. Mutations in complementation groups I and II are unusually frequent in both strains, suggesting that the use of 2 different temperature-sensitive mutants did not affect which aggregation mutants were isolated. However, the distribution of CG I and CG II mutants among *ts119cy^R* mutants isolated after two different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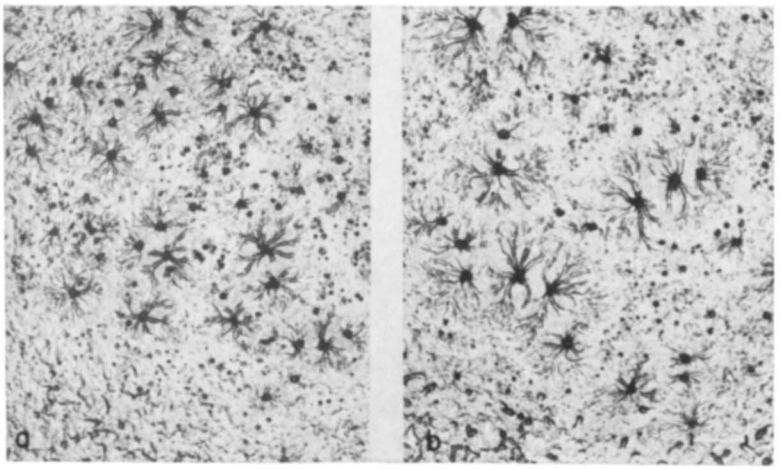

FIGURE 10.—Early stages of aggregation in two ag+ diploids after 38 hours incubation at 21°. (a) *ts119cy^R–ag+*, *ts152–ag+*; (b) *ts119cy^R–A205*, *ts152–936*. Bar represents 2 mm. Cultures standardized as described in MATERIALS AND METHODS.

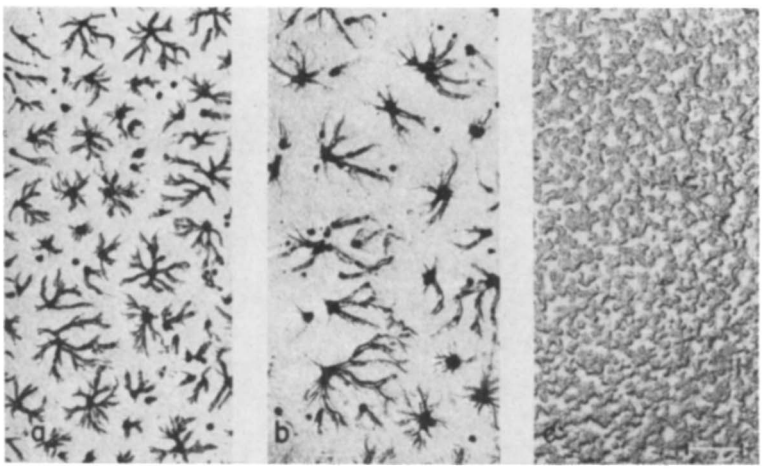

FIGURE 11.—Cultures of two ag+ diploids (a and b) and an ag⁻ diploid (c) after 42 hours incubation at 21°, (a) *ts119cy^R–ag+*, *ts152–ag+*; (b) *ts119cy^R–A205*, *ts152–936*; (c) *ts119cy^R–B515*, *ts152–678*. Bar represents 2 mm. Cultures standardized as described in MATERIALS AND METHODS.

FIGURE 9.—Synergistic development of mixtures of ag⁻ mutants in complementation group I with those not in CG I. Cultures of individual mutants and pairs of mutants were grown for 3.5 days at 21° on NA plates. (a and b) CG I mutants; (a) *ts119cycy^R–B515*, (b) *ts152–936*. (1–4) Non-CG I mutants; (1) *ts152–547*, (2) *ts152–561*, (3) *ts152–98*, (4) *ts119cy^R–A654*. (ab) Mixture of *B515* and *936*. (a1, a2, a3, a4) Mixtures of *B515* with non-CG I mutants. (b1, b2, b3, b4) Mixtures of *936* with non-CG I mutants.

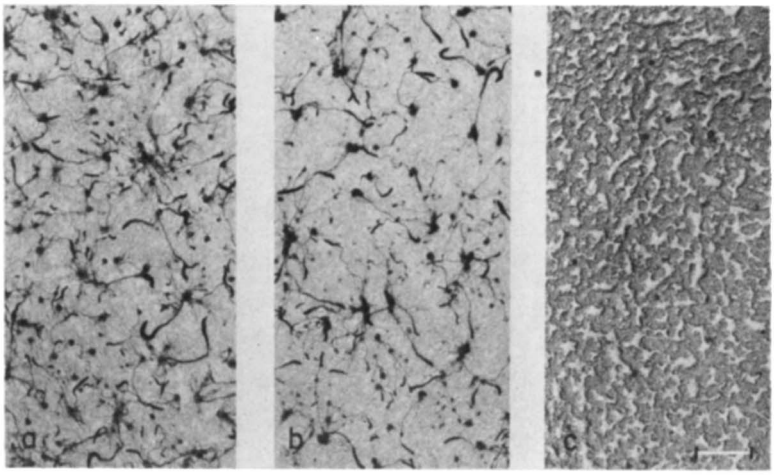

FIGURE 12.—Cultures of two ag+ diploids (a and b) and an ag- diploid (c) after 50 hours incubation at 21°. (a) *ts119cy^{R}-ag+*, *ts152*-ag+; (b) *ts 119cy^{R}-A205*, *ts152-936*; (c) *ts119cy^{R}-B515*, *ts152-678*. Bar represents 2 mm. Cultures standardized as described in MATERIALS AND METHODS.

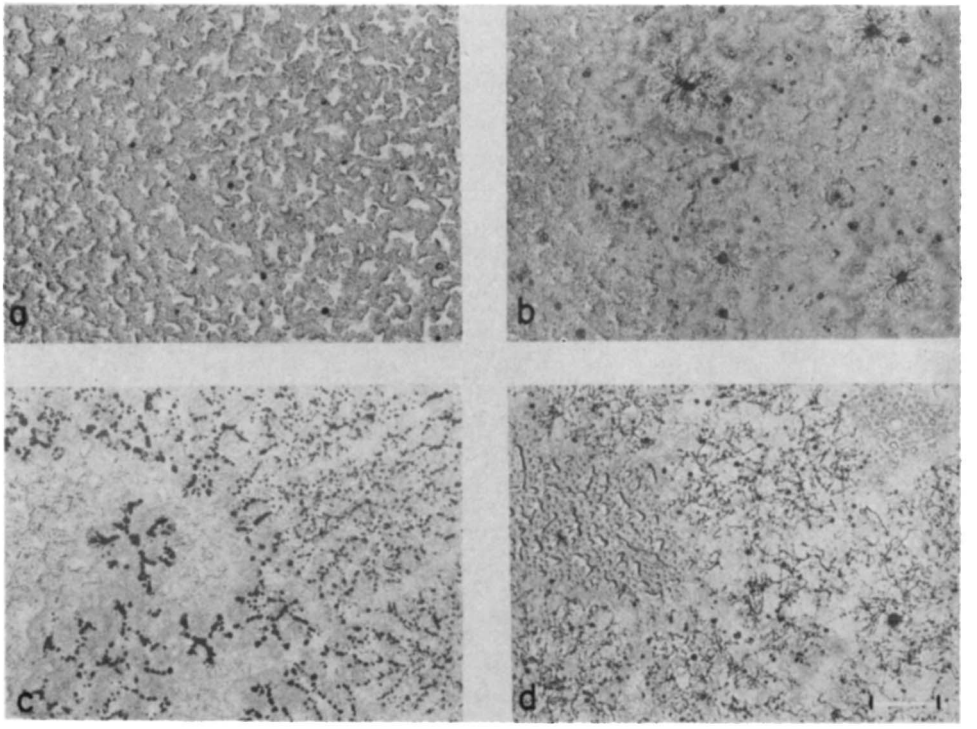

FIGURE 13.—Development of a leaky ag+ diploid, *ts119cy^{R}-B515*, *ts152-678*, after incubation for (a) 50, (b) 65, (c) 75, and (d) 100 hours at 21° Bar represents 2 mm. Cultures standardized as described in MATERIALS AND METHODS.

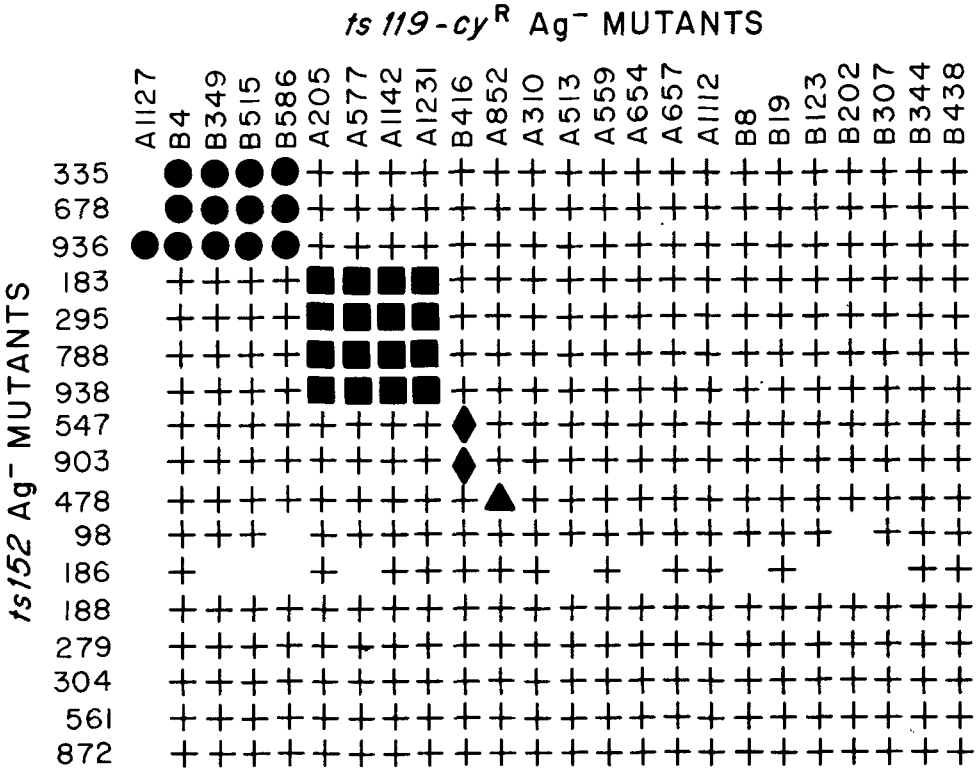

FIGURE 14.—Results of complementation tests performed by isolating diploid cells formed by the fusion of *ts119cy*^{R} ag⁻ mutants and *ts152* ag⁻ mutants. Blank spaces in the matrix refer to mutant pairs for which diploids have not been isolated. A (+) means that the 2 ag⁻ mutations complement in a diploid. Noncomplementing mutants are shown by various symbols indicating different complementation groups: CG I, (●); CG II (■); CG III (◆); CG IV (▲). Many noncomplementing pairs show some interallelic complementation. This figure is taken from WARREN, WARREN and COX 1975.

nitrosoguanidine treatments is nonrandom. There was more than a ten-fold difference in survival (0.4% vs 6%) following these two treatments, perhaps because the cells were in different growth stages. Mutants from the first treatment include only 1 of 5 members of CG I and all 4 members of CG II, while mutants from the second include 4 of 5 in CG I and none in CG II. All of these are independent mutations, and, in addition, most are recognizable from each other by slight phenotypic variations, such as how rounded cell mounds are and whether any fruiting bodies form (see Figure. 5 a–d).

Determination of ploidy: The ploidy of cells thought to be noncomplementing diploids was checked to show that they were not simply temperature-resistant haploid revertants. All the ag⁻ diploids except *ts119cy*^{R}*–A852*, *ts152–478* make at least a few very small fruiting bodies upon prolonged incubation, and their spores were found to be large and abnormally shaped, as diploid spores are known to be (SUSSMAN and SUSSMAN 1962). In addition, all 32 of the ag⁻ diploids were

cycloheximide sensitive, yet segregated cycloheximide resistant cells at frequencies from 5.6×10^{-5} to 3.8×10^{-3}, showing that they were heterozygous for the cycloheximide markers.

Diploid cells grow at 30°, but should segregate temperature-sensitive cells. There is no way to select for temperature-sensitive cells directly, but temperature resistance or sensitivity can be determined in cycloheximide resistant segregants. The frequencies of temperature-sensitive and temperature-resistant cells among 100 or more segregants of each of the 32 ag⁻ diploids were determined; they all segregated temperature-sensitive cells. The average from all ag⁻ diploids was 69 Ts Cells per 100 segregants, which is fairly close to 75, the number expected if the 2 genes for temperature sensitivity segregate independently of each other and independently of the cy^R chromosome and its homolog. It is reasonable that the number of Ts cells is lower than the expected 75% since they grow more slowly than wild-type cells, even at 21°. The range was 53 to 99 Ts cells per 100 segregants. All four ag⁻ diploids containing *A205* gave extremely high frequencies of Ts cells (97–99/100 segregants), while the next highest frequency was 84 Ts cells per 100 segregants.

Aggregation phenotypes of cy^R *segregants*: Since the two ag⁻ mutations in noncomplementing diploids must be on homologous chromosomes, they should segregate parental types only, unless recombination has occurred. The aggregation phenotypes of 100 cycloheximide-resistant clones have been examined for each of 15 ag⁻ diploids. No ag⁺ cells have been found among these segregants, and phenotypes like one or the other parent were most common. But many segregants did develop better than either parent and looked similar to the diploids from which they were derived. The highest frequency with which these abnormal segregants were found was 46/100. Abnormal segregants from one diploid had large, abnormally shaped spores, but those of the others had spores that were not obviously different from haploid spores. The simplest explanation for these abnormal segregants is that they were aneuploids containing both homologs that carry the aggregation mutations in question.

If the unusual segregants are aneuploids, they should segregate out parental aggregation phenotypes. Five such segregants were cloned, and 4 of them segregated out some parental-like cells among 50 clones checked. Figure 15 shows clones derived from one of the unusual segregants of a leaky diploid, *ts119*cy^R–*B349*, *ts152–936*. The original cycloheximide-resistant segregant was cloned, and 50 plaques were picked and spread on small agar plates and incubated 5 days, more than 2 days beyond the time when wild-type cells would have covered the plate with fruiting bodies. Of the 50 clones tested, 12 were clean ag⁻ parental types (Figure 15, type *a*), 24 had small areas of abnormal aggregation up to 1 cm in diameter (Figure 15, type *b*), and 14 had areas of abnormal aggregation and tiny fruiting bodies that covered a total of ¼ to ¾ of the plate (Figure 15, type *c*). A representative of each type was again cloned; type *a* gave 44/50 clean ag⁻ clones, type *b* gave 26/50 clean ag⁻ clones, and type *c* gave 8/50 such clones. Types *a* seem to be cultures in which aneuploids segregated out parental types early in growth so that they formed a large proportion of the population of cells

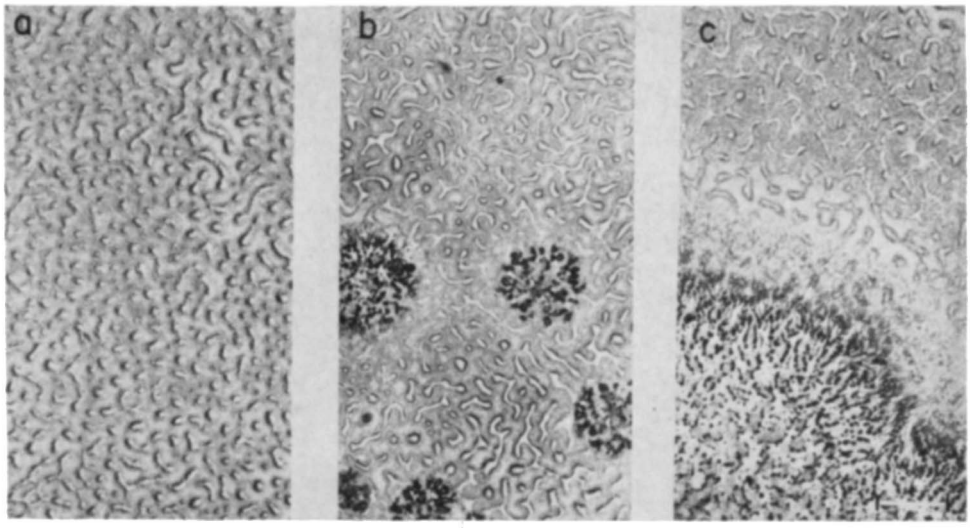

FIGURE 15.—Clones derived from an abnormal cycloheximide resistant segregant of a leaky ag⁻ diploid, *ts119cy^R–B349*, *ts152–936*. Bar represents 2 mm.

on the plate. In type *b* cultures, there were presumably more aneuploids present than in type *a* cultures, but fewer than in type *c* cultures, and these proportions are reflected in the frequencies of different types upon subcloning.

Estimate of the number of complementation units controlling aggregation (ag genes): By making the assumption that the isolation of mutants affected in any of the ag genes is equally probable, it is possible to make statistical estimates of the number of ag genes from the data in Figure 14. The method used here is the maximum likelihood estimator and is discussed in the Appendix. In making these calculations it was assumed that *ts119–cy^R–A1127* (which has been lost) would not complement with *ts152–335* or *678*, since it did not complement with another CG I mutant, *ts152–936* (see Figure 14).

Although the equal probability assumption is necessary in any estimate based on non-saturated sampling, it does not seem to be warranted here. Mutants in complementation groups I and II appear to be abnormally frequent and are probably mutational "hot spots" (see DISCUSSION). Consequently, estimates were made with and without inclusion of CG's I and II. Figure 16 presents graphs of the likelihood of hypothetical numbers of ag genes based an various outcomes of the complementation tests. If all data are used, the resulting curve (a) gives a maximum likelihood estimate of 12. If the two hot spots are excluded from the calculations, the resulting curve (b) gives an estimate of 45 other ag genes, the total estimate being 47. Curve (c), which yields an estimate of $35 + 2$, was calculated by omitting the hot spot data and supposing that one more non-complementing pair of mutants was found upon completion of the remaining complementation tests.

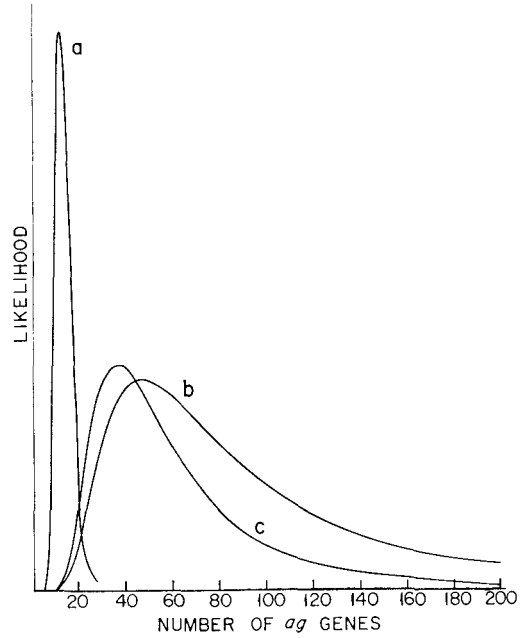

FIGURE 16.—Likelihood curves for three assumptions about the data in Figure 14: Curve (a), all data were used; (b), mutants in CG's I and II were omitted; (c), it was assumed that a single noncomplementing pair would be found in a fifth CG upon completion of the complementation matrix. Details in the text and APPENDIX.

DISCUSSION

We have developed a system for a genetic analysis in *P. violaceum* similar to that used in another cellular slime mold, *D. discoideum* (LOOMIS 1969; KATZ and SUSSMAN 1972). Diploid cells formed by spontaneous fusion of two nonallelic temperature-sensitive haploid cells are selected by incubation at a nonpermissive temperature. One of the temperature-sensitive strains also carries a recessive cycloheximide resistance mutation; thus haploid segregants can be selected from heterozygous clones by plating large numbers of cells on cycloheximide medium (KATZ and SUSSMAN 1972). Evidence for diploidy is spore size (SUSSMAN and SUSSMAN 1962) and the ability of the cycloheximide-sensitive clone to segregate cycloheximide-resistant cells that can be either temperature-sensitive or temperature-resistant. Cycloheximide-resistant segregants sometimes have abnormal morphologies and appear to be aneuploid even though their spores are usually small. Aneuploidy has been suggested as a transient step in the process of haploidization in *D. discoideum* by SINHA and ASHWORTH (1969). More recently, BRODY and WILLIAMS (1974) have verified the existence of aneuploid cells by staining chromosomes.

We have utilized the genetic system described above to perform complementation tests between pairs of aggregation-defective (ag^-) mutants of *P. violaceum*. Ag^- mutants isolated in two different temperature-sensitive strains were

tested for complementation in diploids. If a diploid heterozygote displayed aggregation morphologically and temporally like that of wild-type cells, it was assumed that the 2 ag⁻ mutants were affected in different functions, while abnormal aggregation by such diploids was taken to indicate that the mutants were allelic. Using 24 *ts119cy*^{R} ag⁻ mutants and 17 *ts152* ag⁻ mutants, 380 diploids were isolated of which 32 showed defective aggregation. Noncomplementing mutants define 4 complementation groups; within each complementation group the morphologies of mutants are strikingly similar. In addition, mutants in complementation group I have the unique property of causing aggregation when mixed with non-CG I mutants. Preliminary experiments (A. J. WARREN, unpublished data) suggest that the factor responsible for aggregation can go through a dialysis membrane, although WEBER and RAPER (1971) reported no effect through a dialysis membrane using apparently similar *D. discoideum* mutants.

Interallelic Complementation:

Many diploids containing noncomplementing ag⁻ mutations were found to be "leakier" than either haploid parent; that is, they would eventually form more fruiting bodies upon prolonged incubation. Aggregation in all of these ag⁻ diploids is very different, both morphologically and temporally, from wild-type aggregation, however. The ag⁻ diploid shown in Figure 13 is one of the leakiest isolated and definitely aggregated better than either parental type; nonetheless, its pattern of development is similar to that of another mutant in the same complementation group, *ts152-335* (Figure 6). These results are compatible with the interpretation that two interacting mutant proteins can give rise to a phenotype similar to another allelic mutant protein acting alone. In general, interallelic complementation of this kind is explained by the fact that many enzymes are multimers and that some mutations produce local misfoldings of a protein which can be partially corrected by interaction with an adjacent region that has the correct fold (CATCHESIDE and OVERTON 1958; CRICK and ORGEL 1964; see also FINCHAM 1966).

Mutants that produce proteins with some residual activity are less likely to be highly abnormal in tertiary structure and more likely to be capable of such interaction than other mutants. Thus, the "leakiness" of our mutants may help to explain the high frequency with which we have observed interallelic complementation. Experiments with Neurospora support this possibility (DE SERRES 1963; BROCKMAN and DE SERRES 1963). Also, since amebae remain viable over extended periods of time while a necessary product can slowly accumulate or act, these complementation tests are very sensitive. In another very sensitive test (burst size), BERNSTEIN, DENHARDT and EDGAR (1965) found interallelic complementation to be very frequent among pairs of temperature sensitive T4 mutants.

The estimate of the number of complementation units affecting aggregation (ag genes): The basic and necessary assumption in the statistical analysis, that all ag genes have an equal probability of being represented among our mutants, does not seem to hold. Mutations in complementation groups I and II are abnor-

mally frequent; diploids formed from pairs of these mutants account for 29 of 32 ag⁻ diploids. The other 2 complementation groups, III and IV, account for the other 3 ag⁻ diploids.

If we assume for a moment that genes corresponding to complementation groups I and II are not mutational "hot spots", the estimate for the number of ag genes is 12. Then the 7 *ts152* and 13 *ts119cy*^{R} ag⁻ mutants not in complementation groups I–IV must be distributed among about 8 complementation groups in such a way that mutants in a given complementation group are all in one temperature-sensitive strain. Thus, when we assume complementation groups I and II are representative of all ag genes, we force all other complementation groups to be distributed in a very nonrandom way between the two strains.

The estimate of 47 ag genes results if mutants in CG's and I and II are deleted and then added back to the resulting estimate. This estimate is based on very little data and consequently is not very precise. It would fall from 47 to 37 if one more noncomplementing pair of mutants were found upon completing the remaining complementation tests. This sensitivity to small changes in the data and the broad peaked character of the likelihood curve of Figure 16(b) are both manifestations of the uncertainty of the estimate. A more quantitative assessment of the estimate could have been made by calculating confidence intervals. However, this would be misleading since the calculations are still based on the assumption that mutants in CG's III and IV are recovered with frequencies representative of most ag genes; that is, that they are not mutational "warm spots." Any range in apparent mutability will lead to an underestimate of the total; thus the estimate of approximately 50 genes must be considered an underestimate.

This conclusion should be qualified; if many of our mutants have general metabolic defects rather than specific aggregation defects, then this would lead to an overestimate of the number of ag genes. Since the data in Table 1 show that most of the mutants have normal generation times, we believe that the effect of this factor on the estimate will be small.

Does the estimate of 50 ag genes seem reasonable? FIRTEL (1972) has some evidence in *D. discoideum*, a cellular slime mold similar to *P. violaceum*, that there are RNA transcripts complementary to enough unique DNA sequences to determine about 16,000 proteins. One to four thousands of these are present only during the time of aggregation. If the unique transcript data can be confirmed, then our results suggest that aggregation does not require a large fraction of these genes, if we identify complementation units with single gene transcripts.

In the yeast *Saccharomyces cerevisiae*, a simple eukaryote with about one-third to one-half as much DNA as *D. discoideum* (OGUR *et al.* 1952; HARTWELL 1970; FIRTEL and BONNER 1972), complementation studies of mutants suggest that sporulation (meiosis and ascospore formation) involves about 50 genes (ESPOSITO *et al.* 1972) and mitosis involves somewhat more than 30 genes (HARTWELL *et al.* 1973). It is difficult to relate *a priori* the complexities of these types of events to aggregation, but these results do suggest that they are of the same order of genetic complexity. In all three cases, then, major events in the life cycle seem to require a small number of complementation units. However, the

amount of DNA these complementation units represent remains unknown. In some other fairly simple eukaryotes estimates of gene number are more than an order of magnitude lower than would be predicted from the amounts of unique DNA—*e.g.*, in *Drosophila melanogaster* (JUDD, SHEN and KAUFMAN 1972; HOCHMAN 1971; RUDKIN 1965) and in the nematode *Caenorhabditis elegans* (BRENNER 1974; SULSTON and BRENNER 1974).

Another way to evaluate our estimate is to try to assess the phenotypic complexity of aggregation. In this study, mutants were selected which were unable to stream toward a center or to form the tightly adhering lumps of cells characteristic of normal aggregation. Defects may be in the ability to produce or secrete the attractant properly, to detect it, or to respond to it properly, that is to move toward the signal and relay it. Mutant cells may also be defective in one of the surface sites necessary for adhesion. It is unlikely that ag⁻ mutants would include non-motile cells since they would probably be unable to capture bacteria, but mutants unable to move in a directed manner may be included. From what is known about aggregation, it seems intuitively reasonable that it could be carried out by a number of proteins approximately equal to the number of complementation units estimated (see WARREN 1974, for discussion). However, we will not know until all components of aggregation are understood on a biochemical level.

Note added in proof: Recently, similar studies with *D. discoideum* have been published [COUKELL, M. B. (1975) Molec. Gen. Genet. **142**: 119–135; WILLIAMS, K. L. and P. C. NEWELL (1976) Genetics **82**: 287–307].

APPENDIX
MAXIMUM LIKELIHOOD ESTIMATOR

The method used to form the estimate $\hat{n}$ of the number of ag genes n will be discussed. The likelihood that a hypothesis is true is defined to be equal to the probability of obtaining the experimental outcome which has been observed, calculated on the assumption that the hypothesis in question holds. In the present case the hypotheses considered are $n=1$, $n=2$, and so forth; the maximum likelihood estimator $\hat{n}$ corresponds to the hypothesis with the greatest likelihood (CRAMÉR 1946).

The experiment consisted of crossing m mutants isolated in one strain (*ts119 cy*^{R}) with m' mutants isolated in another strain (*ts152*). The outcome may be summarized by the set of numbers $n_{11}, n_{12}, \ldots, n_{mm'}$, where n_{ij} is the number of complementation groups with i *ts119 cy*^{R} mutants and j *ts152* mutants. The assumption that mutants of each ag gene were isolated independently and with equal probability makes it possible to calculate the probability of the observed outcome using combinational methods. This probability is given by

$$\frac{m!m'!}{(m-\mu)!(m'-\mu')!(1!1!)^{n_{1,1}}\ldots(m!m'!)^{n_{m,m'}}} \times \sum_{j=0}^{n-\nu} \frac{n!}{(n-\nu-j)!n^{m+m'}} \sum_{i=0}^{j} S_{m-\mu}^{(i)} S_{m'-\mu'}^{(j-i)},$$

where

$$\mu = \sum_{i=1}^{m} \sum_{j=1}^{m'} i\, n_{ij},$$

$$\mu' = \sum_{i=1}^{m} \sum_{j=1}^{m'} j\, n_{ij},$$

$$\nu = \sum_{i=1}^{m} \sum_{j=1}^{m'} n_{ij},$$

and

$$S_k^{(s)} = \sum_{\substack{n_1+n_2+\ldots+n_k=s \\ n_1+2n_2+\ldots+kn_k=k}} \frac{k!}{(1!)^{n_1} n_1! \ldots (k!)^{n_k} n_k!};$$

$S_k^{(s)}$ is Stirling's number of the second kind (ABRAMOWITZ and STEGUN (1964).

A computer program was written to evaluate the above expression for a series of values of n. That value which yielded the largest likelihood was taken to be $\hat{n}$.

A similar approach has been used elsewhere in a somewhat less complicated situation (LEWONTIN and PROUT 1956).

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