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Effects of Specific Cations on Aggregation and Fruiting Body Morphology in the Myxobacterium *Stigmatella aurantiaca*

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The cation requirements for fruiting body formation in the myxobacterium *Stigmatella aurantiaca* on agarose were determined. Calcium alone caused the cells to aggregate into interconnecting ridges. Under these conditions, stalk formation was severely depressed but sporangia frequently formed. The combination of magnesium and manganese was necessary for optimal formation of discrete aggregates (rather than ridges) and stalks. Manganese inhibited sporangium development. The inclusion of calcium into the magnesium-manganese medium overcame the inhibition by manganese and stimulated the production of multiple sporangia.

The myxobacteria are procaryotes that are particularly suitable for studying the molecular bases for cell interactions and directed cell motility. The cell interactions are complex and known to include cell-to-cell signaling and cohesion between developmentally competent cells (5; unpublished data). The movement of the cells during fruiting body formation has no known parallel among procaryotic organisms. Its relationship to the sensing and signal transduction system that underlies ordinary bacterial chemotaxis is not known (10). We have recently devised conditions for inducing synchronous development of *Stigmatella aurantiaca* on Difco agar or in liquid suspension (6, 7). This should facilitate investigations into the molecular mechanisms that are responsible for the multicellular shapes that myxobacteria produce during the construction of morphologically complex fruiting bodies. The morphogenetic movements of the cells can be viewed as occurring in three sequential stages that culminate in the production of fruiting bodies (4, 6–8). The first stage consists of localized accumulations of cells into discrete multicellular aggregates resulting from movement into numerous collection centers. We found that this phase of development is markedly stimulated by light, guanine derivatives, and a diffusible morphogen produced by the cells (6, 7, 11, 12). Shortly after each aggregate forms, the cells move upward and construct a stalk. The third stage is the partitioning of the cell population at the tip of the stalk into multiple sporangia. Another form of development, one that usually occurs in the dark, is typified by aggregation of cells into massive ridges that frequently interconnect (6, 7). Sporangia can form when ridge formation takes place, but the sporangia are usually sessile and rest directly on the ridges rather than on stalks.

To facilitate further research with *S. aurantiaca*, we report here the conditions for obtaining optimal fruiting on agarose, a medium which is more highly purified than agar (1, 9). The use of media substantially free of contaminating organic matter such as amino acids can be of use in pursuing nutritional and biochemical studies of the fruiting process (2). We have used the agarose media to investigate the cation requirements for the different morphological stages of development. We found that three cations, i.e., calcium, magnesium, and manganese, are necessary for the formation of fruiting bodies possessing stalks and multiple sporangia. The omission of any of these cations results in drastic changes in either aggregation or fruiting body morphology and in particular can shift the pattern to interconnecting ridges even in the light.

MATERIALS AND METHODS

Organism and cultivation. *S. aurantiaca* DW4 was used for all experiments. This is a dispersed growing variant that we isolated from strain CCf (6). It can be subcultured repeatedly in liquid without losing the ability to form fruiting bodies when starved on an agar surface under conditions that we described (6, 7). The cells were grown in Casitone (Difco) in shaking liquid culture (6).

Induction of fruiting body formation. Development was induced on 1% agarose or 1.5% Difco agar containing 10 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.1. To determine the cation requirements, 3 ml of agar was pipetted into plastic petri dishes, 3.5 by 1 cm, containing a concentrated solution (0 to 180 μl) of the chloride salts to be tested. The petri dishes were left uncovered while the agar solidified. They were then covered and stored at room temperature for 20 to 24 h before use. Cells were harvested from the growth medium when they reached a density of from 1.1×10^8 to 1.4×10^8/ml and were washed once with a solution of 3.4 mM $CaCl_2$ and 10 mM each of KCl and NaCl (CPS solution). The cells

were then resuspended in CPS to a density of 4×10^{10}/ml, and a 5-μl drop was placed in the center of the petri dish. When the liquid had soaked into the agar, the plates were covered and incubated at 30°C in 215 lx of incandescent light. We previously showed that light is essential for consistent fruiting (6, 7). Aggregates, fruiting bodies, and sporangia were counted with the aid of a dissecting microscope. Control experiments demonstrated that washing and suspending the cells in CPS did not influence the cation requirements that we observed.

Scanning electron microscopy. Small blocks of agar containing fruiting swarms were mounted on specimen stubs, air-dried overnight, and metal-coated in a vacuum evaporator. The first coat was carbon, followed by a mixture of gold and palladium (60:40). Specimens were examined with an ETEC Corp. Autoscan Scanning Electron Microscope.

Reagents. Agarose (electrophoresis grade) was purchased from ICN Pharmaceuticals, Inc., Cleveland, Ohio. HEPES was a product of Sigma Chemical Co., St. Louis, Mo. For some experiments, ME agarose purchased from Miles Laboratories, Elkhart, Ind., was used.

RESULTS

Fruiting on Difco agar. As reported earlier by Grilione and Pangborn (4) and later by Qualls et al. (6, 7), *S. aurantiaca* will form fruiting bodies on Difco agar supplemented with CPS. The fruits have two to four sporangia (usually) and a stalk that is approximately 90 μm tall in samples that have not been dried (Fig. 1A). With strain DW4 and conditions as reported from this laboratory, development was synchronous and complete within 24 h. We were not able to demonstrate an absolute requirement for a specific cation by using Difco agar. A small and variable number of fruiting bodies formed in the absence of added cations. The addition of the

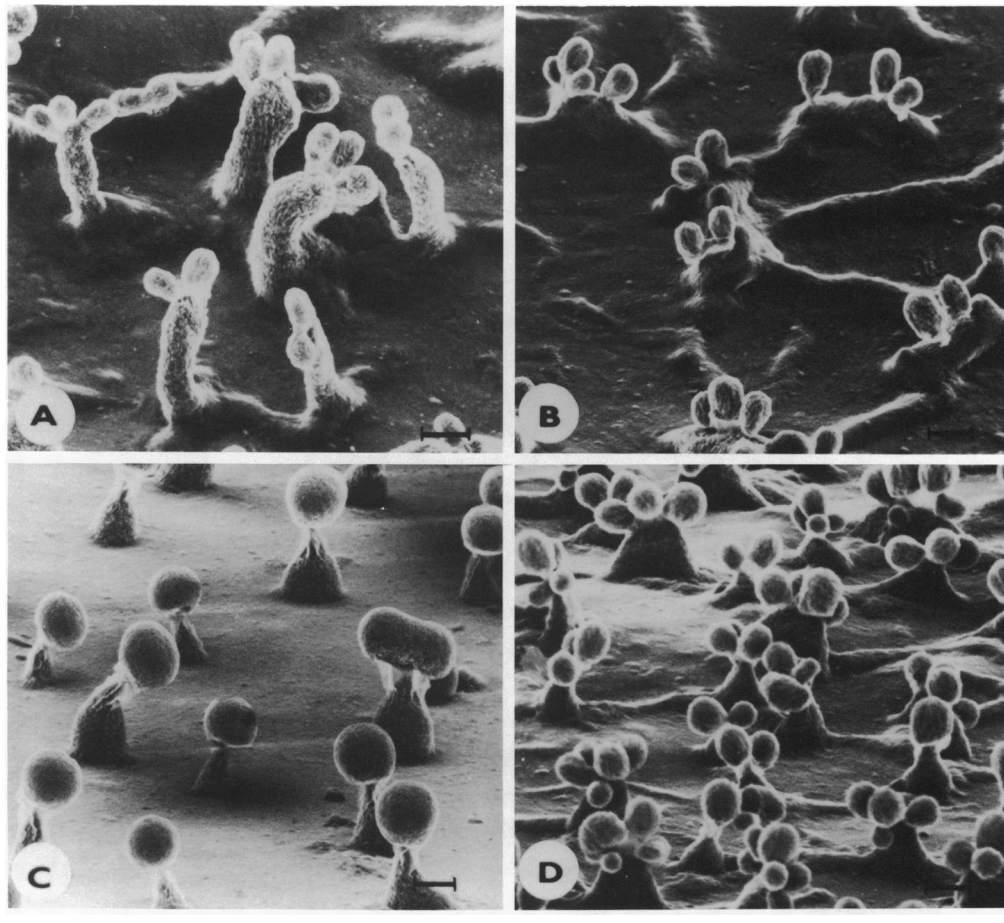

FIG. 1. *Scanning electron micrographs of fruiting bodies formed on different media. Development had proceeded for 48 h in all samples. (A) Difco agar supplemented with 3.4 mM $CaCl_2$, 10 mM NaCl, and 10 mM KCl (CPS). Bar, 40 μm. (B) Agarose supplemented with 3.4 mM $CaCl_2$. Bar, 40 μm. (C) Agarose supplemented with 2 mM $MgCl_2$ and 1 mM $MnCl_2$. Bar, 25 μm. (D) Agarose supplemented with 2 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.2 mM $CaCl_2$. Bar, 43 μm.*

chloride salts of sodium and potassium, or magnesium, calcium, or manganese, stimulated the production of fruiting bodies severalfold.

Fruiting on agarose. Because of the likelihood of contaminating amino acids and cations in Difco agar (2), we investigated cation requirements by using agarose. All of the experiments for which data are presented were performed using ICN agarose. We experienced minor variations in results using agarose with a different lot number, and this will be pointed out when pertinent. We obtained similar data on cation requirements using ME agarose from Miles Laboratories, but the manganese ion concentration had to be reduced to 0.3 to 0.4 mM because higher concentrations were inhibitory. We found that manipulation of three cations, i.e., calcium, magnesium, and manganese, could determine whether the cells formed ridges, discrete aggregates, stalks, or multiple sporangia.

Calcium. When calcium was the sole cation added, the cells aggregated into massive, interconnecting ridges. Frequently sporangia developed, but the extent of sporangium formation varied with the lot number of the agarose. Of importance is the fact that stalk formation was severely depressed when calcium was the only cation present, and the sporangia then usually rested directly on the ridges (Fig. 1B). When stalks were present they represented approximately 20% or fewer of the sporangial clusters. We occasionally observed short pedicles. These did not resemble stalks but rather the noncellular coat that surrounds the fruiting bodies (Fig. 2). We were sometimes able to increase the frequency of stalks by adding 10 mM each of sodium and potassium chloride, but this also depended upon the lot number of ICN agarose and did not occur at all when we used ME agarose from Miles Laboratories. As a measure of the concentration of calcium required for development, we counted clusters of sporangia (which usually rested directly on the ridges) as a function of calcium ion concentration. As shown in Table 1, the optimal calcium ion concentration extended from approximately 2 to 3 mM to $\geq$10 mM.

Magnesium. The cells responded differently to magnesium. Ridge formation was less pronounced, and aggregates were discrete, although sometimes elongated. Stalk formation was also more prevalent in the magnesium medium, although this also varied with the agarose used. For many experiments, most of the fruits were supported by very short stalks, and it was possible to count fruiting bodies as a function of the magnesium ion concentration. The data summarized in Table 1 indicate an optimum between 2 and 4 mM and inhibition by higher concentrations.

Positive cooperativity with manganese. The combination of manganese and magnesium

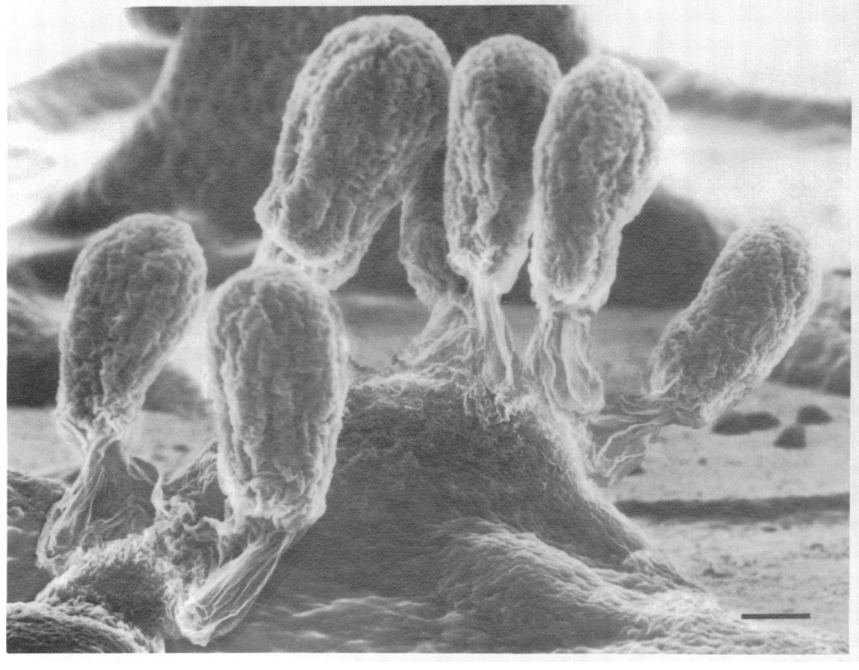

FIG. 2. *Sporangia supported by pedicles formed on media containing 3.4 mM $CaCl_2$. Bar, 17 μm.*

TABLE 1. *Stimulation of development by magnesium and calcium ions*

Concn of cation (mM)	Development[a]	
	$MgCl_2$ (No. of fruiting bodies per 0.2-mm^2 spot)	$CaCl_2$ (No. of clusters of sporangia)
0	0	0
0.1	4 ± 7	ND
0.2	20 ± 16	39 ± 29
0.4	50 ± 45	53
0.5	50 ± 40	47
0.6	ND	50 ± 40
0.8	110	ND
1.0	110 ± 25	67 ± 13
2.0	131 ± 36	165 ± 63
3.4	151 ± 29	252 ± 43
4.0	102 ± 35	167 ± 32
5.0	103 ± 43	ND
6.5	96	ND
8.0	76 ± 38	230 ± 29
10.0	6 ± 9	315

[a] In the magnesium medium, fruiting bodies with stalks formed. In the calcium medium, approximately 80% of the clusters of sporangia were on ridges. ND, Not done. Plus and minus values refer to one standard deviation.

had remarkable results on fruiting body formation. The numbers of fruiting bodies were always highest when both cations were present, and very importantly, all the fruiting bodies had tall stalks (Table 2, Fig. 1C). Similar results were obtained using ME agarose, provided the manganese concentration was lowered to 0.3 to 0.4 mM. Ridge formation was always minimal in the presence of manganese. Manganese also interacted cooperatively with calcium to promote aggregation and stalk formation, but only over a narrow range of manganese concentration (0.2 to 0.3 mM), owing perhaps to the fact that calcium was unable to prevent manganese toxicity (data not shown).

Opposing effects of cations on sporangium development. The effect of manganese on sporangium formation was opposite to its stimulatory influence on aggregation and stalk development. As shown in Table 3, 1 mM manganese typically decreased the number of fruiting bodies with two or more sporangia from approximately 90% to about 40%. The greatest change occurred in the class of fruiting bodies with three or more sporangia, in which manganese decreased the frequency from 50 to 11%. The percentage of monosporangial fruiting bodies increased from 17 to 59% when manganese was added. Calcium completely reversed the inhibition by manganese. We observed similar effects of manganese and calcium when using agarose from Miles Laboratories but at 0.4 mM manganese. With lower concentrations of manganese, the number of bisporangial fruiting bodies was higher (data not shown). Neither magnesium, sodium, nor potassium chloride over a wide concentration range could substitute for calcium in increasing the numbers of sporangia in the magnesium-manganese medium (data not shown). The effect of calcium on sporangium development can be seen in Fig. 1B, C, and D.

Effect of other cations. We also tested barium, zinc, and lithium chlorides over a wide concentration range (0.2 mM to 20 mM). Zinc caused cell lysis and therefore inhibited fruiting body formation even at concentrations as low as 3 μM. These concentrations of zinc were 100 times lower than those required to inhibit vegetative growth in liquid suspension. Barium or lithium did not promote fruiting body formation, although, as with potassium or sodium, they frequently stimulated ridge formation when present in high concentrations (over 5 mM).

TABLE 2. *Cooperative interaction between manganese and magnesium on fruiting body formation*[a]

$MnCl_2$ concn (mM)	No. of fruiting bodies at $MgCl_2$ concn (mM):	
	0	2.0
0	0	131 ± 36
0.1	8 ± 16	177
0.2	13 ± 13	170 ± 33
0.3	20 ± 24	239 ± 69
0.4	8 ± 12	220 ± 38
0.5	0	295 ± 38
1.0	0	301 ± 55

[a] Fruiting bodies per spot. Each spot was 0.2 mm^2. Plus and minus values refer to one standard deviation.

TABLE 3. *Effect of cations on number of sporangia per fruiting body*[a]

No. of sporangia	% of total fruiting bodies having indicated no. of sporangia in medium containing:		
	$MgCl_2$[b]	$MgCl_2$, $MnCl_2$[c]	$MgCl_2$, $MnCl_2$, $CaCl_2$[c]
1	17 ± 8	59 ± 7	11 ± 5
2	39 ± 15	31 ± 6	30 ± 6
3 or more	50 ± 28	11 ± 4	80 ± 10
4 or more[d]	17	3	24

[a] Concentrations of cations: $MgCl_2$, 2.0 mM; $MnCl_2$, 1.0 mM; $CaCl_2$, 0.2 mM. Manganese increased the number of fruiting bodies approximately twofold. Calcium did not affect the total number of fruiting bodies significantly.

[b] The results of two experiments with duplicate samples.

[c] The results of three experiments with duplicate samples.

[d] The results of one experiment with duplicate samples.

Synchrony of development. The data thus far presented demonstrate that a mixture of 2.0 mM magnesium, 1.0 mM manganese, and 0.2 mM calcium chloride (CMM) promoted extensive fruiting on agarose. Under these conditions the cells developed synchronously, although 14 to 15 h later than cells developing on Difco agar in CPS (Fig. 3).

DISCUSSION

Essentially, our observations were that calcium ion (or high concentrations of monovalent cations) caused the cells to aggregate into ridges, whereas a combination of magnesium and manganese ion promoted the formation of discrete aggregates and stalks. It thus appears as though certain events in the formation of aggregates and stalks require both magnesium and manganese. In the magnesium-manganese medium, sporangium formation was depressed owing to an inhibition by manganese. Under these circumstances the addition of a small amount of calcium greatly stimulated the production of multiple sporangia. Therefore, there may be stages associated with the final partitioning of the cells into multiple sporangia that are antagonized by manganese and stimulated by either calcium or a mixture of calcium and magnesium. However, the data do not distinguish between direct effects of the cations on enzymatic reactions critical to aggregation, stalk, or sporangium formation, and indirect influences on development due to cation-induced changes in the general physiology of the cells.

The use of agar or agarose as a solidifying agent for experiments in which cation requirements are determined requires some comment. There are inherent difficulties associated with the use of agar. This is due to the fact that agar commercially sold to solidify media contains considerable amounts of agaropectin, a sulfated galactose polymer (1). The sulfate groups pose a problem because they can bind cations and hence contribute uncertain quantities to the known concentrations of free cations added to the media. It is known that commercial preparations of agar contain significant amounts of calcium and magnesium, some of which may have been introduced during the washing procedure (3). Agarose has a much lower sulfate content, and the purification procedures for agarose probably lower the amount of contaminating salts in the final product (1, 9). However, even agarose can vary considerably in sulfate content, according to the manufacturer's specifications. During the course of these experiments we did test other kinds of agarose as well as ICN agarose with a different lot number. When we used ICN agarose with a different lot number we found essentially the same cation effects as reported here. However, there were some minor differences. When added singly, calcium ion was less effective in promoting sporangia development (although ridges still formed), and magnesium did not produce as many stalks. On ME agarose supplied by Miles Laboratories, fruiting was poor on either CPS or CMM. However, by lowering the manganese concentration to 0.3 to 0.4 mM we were able to reproduce responses to magnesium, manganese, and calcium similar to those on ICN agarose.

We initiated these experiments to find whether development showed an absolute specificity for calcium ion, since this was the only divalent cation added to Difco agar in the earlier reports by Grilione and Pangborn (4) and Qualls et al. (6, 7). We found, instead, multiple divalent cation requirements. During the course of these experiments we also established a procedure by which one can study synchronous development of *Stigmatella* in a medium relatively free of contamination with organic nutrients, and we have introduced new options for perturbing development by manipulating the cations.

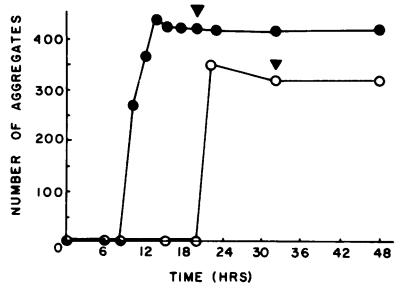

FIG. 3. *Appearance of aggregates as a function of time either on Difco agar supplemented with CPS (●) or on agarose and CMM (○). Arrow indicates when morphologically mature fruiting bodies appeared.*

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LITERATURE CITED

1. **Araki, C.** 1958. Seaweed polysaccharides, p. 15–30. *In* M. L. Wolfrom (ed.), Proc. 4th Int. Congr. Biochem., Vienna, vol. 1. Pergamon Press, New York.
2. **Bretscher, A. P., and D. Kaiser.** 1978. Nutrition of *Myxococcus xanthus*, a fruiting myxobacterium. J. Bacteriol. **133**:763–768.
3. **Bridson, E. Y., and A. Brecker.** 1970. Design and formulation of microbial culture media, p. 229–295. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3A. Academic Press, Inc., New York.
4. **Grilione, P. L., and J. Pangborn.** 1975. Scanning electron microscopy of fruiting body formation by myxobacteria. J. Bacteriol. **124**:1558–1565.

5. **Kaiser, D., C. Manoil, and M. Dworkin.** 1979. Myxobacteria: cell interactions, genetics, and development. Annu. Rev. Microbiol. **33**:595–639.
6. **Qualls, G. T., K. Stephens, and D. White.** 1978. Morphogenetic movements and multicellular development in the fruiting myxobacterium, *Stigmatella aurantiaca*. Devel. Biol. **66**:270–274.
7. **Qualls, G. T., K. Stephens, and D. White.** 1978. Light-stimulated morphogenesis in the fruiting myxobacterium *Stigmatella aurantiaca*. Science **201**:444–445.
8. **Reichenbach, H.** 1974. *Chondromyces apiculatus* (Myxobacteriales). Schwarmentwicklung und Morphogenese, *In* G. Wolf (ed.), Encyclopaedia Cinematographica Film E779/1965. Institut für den Wissenschaftlichen Film, Göttingen.
9. **Russel, B., T. H. Mead, and A. Polson.** 1964. A method of preparing agarose. Biochim. Biophys. Acta **86**:169–174.
10. **Springer, M. S., M. F. Goy, and J. Adler.** 1979. Protein methylation in behavioral control mechanisms and in signal transduction. Nature (London) **280**:279–284.
11. **Stephens, K., and D. White.** 1980. Morphogenetic effects of light and guanine derivatives on the fruiting myxobacterium *Stigmatella aurantiaca*. J. Bacteriol. **144**:406–412.
12. **White, D., W. Shropshire, Jr., and K. Stephens.** 1980. Photocontrol of development by *Stigmatella aurantiaca*. J. Bacteriol. **142**:1023–1024.