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Prestalk and prespore differentiation in *Dictyostelium* as detected by cell type-specific monoclonal antibodies

(pattern formation/cell contact/immunocytochemistry/slime mold/*Dictyostelium discoideum*)

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ABSTRACT **Monoclonal antibodies specifically reactive against prestalk and prespore cells of the cellular slime mold *Dictyostelium discoideum* were obtained. By the use of these antibodies, we examined processes of differentiation of the two cell types during development. Cells stained with prespore-specific antibodies first appeared after 12–14 hr of starvation within cell aggregates with tips, coincidentally with the appearance of other prespore markers. The number of prespore cells then increased to a level of 70–80% of total cells at the slug stage. By contrast, cells stained with prestalk-specific antibodies began to appear after 3 hr of starvation and thereafter increased in number to a maximum of *ca.* 80% after 12 hr of starvation. Stained cells appeared at random in the aggregation field and were not morphologically distinguishable from unstained cells. Furthermore, cells showed a considerable heterogeneity in the amount of antigen they contain. Concomitantly with the increase in prespore cells, the number of cells stained by the prestalk antibodies decreased to a level of *ca.* 20% by the slug stage. From these experiments, we suggest that the prestalk antigen is synthesized in the majority of cells during the early period of aggregation. Within tight cell aggregates, some of these cells lose the antigen to become prespore cells and the normal proportion between the two cell types will eventually result within slugs.**

The cellular slime mold *Dictyostelium discoideum* is well suited for the studies of cell differentiation and pattern formation because of its unique developmental characteristics. After cessation of feeding, *D. discoideum* cells aggregate to form slug-shaped cell masses, which eventually construct fruiting bodies consisting of spores and stalk cells. During the formation of a fruiting body, the anterior cells of a slug differentiate into stalk cells, whereas the posterior cells differentiate into spores.

Recent studies on changes in protein and mRNA syntheses during the development of this organism indicate that the major changes in gene expression occur at the late aggregation stage when tight cell-to-cell contacts form (1–3). This coincides in time with the appearance of prespore cells, as identified by their specific products, such as prespore antigen (4), UDP-galactose–polysaccharide transferase responsible for the synthesis of the antigen (5), and the prespore vacuole (PSV) (6, 7) containing the prespore antigen (8). Prespore and prestalk cells are characterized by the synthesis of specific proteins (9, 10).

By the use of polyspecific antiserum produced against spores [including antibodies reactive against an acid mucopolysaccharide (11)], prespore differentiation has been examined in detail (12–14): prespore cells begin to appear within an aggregate about to form a tip and then rapidly increase in number, so that the prestalk–prespore pattern is completely formed at the standing slug stage. On the other hand, the time and location of prestalk differentiation and its relation to prespore differentiation remain to be solved, as no cellular markers for prestalk differentiation have been known, except for acid phosphatase II (15).

To overcome this difficulty, we tried to obtain monoclonal antibodies that specifically react against either prestalk or prespore cells. Having obtained such antibodies, we examined the processes of differentiation of the two cell types. It was found that prestalk antigens are synthesized in the majority of cells during the early aggregation period, whereas prespore antigens first appear at the late aggregation stage.

MATERIALS AND METHODS

Organisms and Culture. *D. discoideum* NC 4 and a mutant isolated therefrom, dev 1515, were used. The latter shows alternative pathways of differentiation into spores or stalk cells depending on culture conditions (16). NC 4 was grown with *Escherichia coli* in a shaking culture containing a nutrient medium (2% peptone and 2% glucose in 20 mM phosphate buffer, pH 6.4). The cells were collected at the stationary growth phase, washed free of bacteria, and plated either on nonnutrient agar (2%) or Millipore filters saturated with Bonner's standard solution (ref. 17). dev 1515 was grown with *E. coli* in a medium containing 0.5% peptone, 0.5% lactose, and 100 mM mannitol. Washed cells were plated on nonnutrient agar, where the majority of the cells eventually differentiated into stalk cells (16). Disaggregation of slugs and aggregates was conducted either chemically in a solution of Pronase and dimercaptopropanol (18) or mechanically by pipetting in lower pad solution (19). Disaggregated cells were incubated to dedifferentiate, as described previously (20).

Antigen Preparation. Aggregates of dev 1515 were collected when about half of the cells became mature stalk cells. The cells were fixed in absolute methanol at −20°C for 10 min, resuspended in phosphate-buffered saline at pH 7.2, at 1×10^8–10^9 cells per ml, and injected as the antigen. NC 4 cells disaggregated from slugs were also used as an antigen after being resuspended in phosphate-buffered saline without fixation.

Hybridoma Screening. Hybridoma cell lines were isolated essentially as described by Galfre *et al.* (21), with minor modification. Briefly, 6-wk-old female BALB/c mice were injected intraperitoneally with 0.1 ml of cell suspension described above. The injections were repeated three times at 2-wk intervals. The spleen was removed 3 days after the final injection and 1×10^8 spleen cells were fused with 1×10^8 mouse myeloma cells (X63·Ag8) by the use of polyethylene glycol 1540 (Wako). Supernatants from wells were tested for the presence of antibody

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Abbreviation: PSV, prespore vacuole.

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Table 1. Immunofluorescent staining characteristics of monoclonal antibodies

Antibodies	Cell type specificity	Cells: Vegetative	Cells: Aggregative	Cells: Slug: Prespore	Cells: Slug: Prestalk	Cells: Mature spore	Cells: Mature stalk	Cytological location of staining	Injected antigens
B6	Prespore	−	−	+	−	−	−	Cytoplasmic granules	dev 1515 aggregates
SB5	Prespore	−	−	+	−	+	−	Extra- and intracellular	NC 4 slugs
C1	Prestalk	−	+	−	+	−	+	Cytoplasmic granules	dev 1515 aggregates
D4	Prestalk	−	+	−	+	−	+	Cytoplasmic granules	dev 1515 aggregates

The presence and the absence of staining is denoted by + and −, respectively.

against NC 4 cells by the use of the enzyme immune assay method. Peroxidase (Sigma, type VI)-conjugated rabbit anti-mouse Igs were prepared and slug or vegetative cell extract (1 mg of protein per ml) was used for test antigen. The cell extracts were prepared by solubilizing cells in 10 mM Tris·HCl (pH 7.2) containing 1% $NaDodSO_4$ and then were dialyzed against the buffer for 2 days. The wells positive for slug cell extract but not for vegetative cell extract were cloned by limiting dilution at least three times. The clones were tested for cell type specificity by staining sections of migrating slugs (see below).

Indirect Immunofluorescence Staining. Washed cells were incubated either on a cover glass in a moist chamber or in a liquid shake culture and then spread on a cover glass. Cells were fixed in methanol and completely dried. They were treated with supernatant of cloned hybridoma for 2 hr and, after rinsing, with 40× diluted fluorescein-isothiocyanate-conjugated rabbit anti-mouse IgG (Miles-Yeda, Rehovot, Israel) for 1 hr and then rinsed with three changes of phosphate-buffered saline for 10 min each. Because a control experiment showed that the rabbit IgG was nonspecifically bound to NC 4 cells, it was absorbed beforehand: fixed 1×10^7 slug cells were added to 0.1 ml of the IgG solution and kept overnight before being centrifuged.

Slugs were embedded in paraffin and sectioned as described (22). The sections were stained as described above, except that more concentrated IgG solution (10× dilution) was used. The stained preparations were observed under a Nikon fluorescence microscope (model XFEF). Control experiments were conducted with supernatant of unfused myeloma cells, which gave almost no staining in any case.

RESULTS

Antibody Production of Hybridoma. Hybridoma cell lines were screened for the production of antibody reactive against *D. discoideum* NC 4 cells, as described in the preceding section. On an average, about 18% of the wells showed positive reaction on the first assay, but only 20–30% of these gave rise to stable populations of antibody production. Among them, 23% produced stage-specific antibodies, which reacted against slug cell extract but not against vegetative cell extract. After cloning these hybridomas, we tested the cell type specificity of the antibodies they produced and obtained two prestalk- and two prespore-specific clones (Table 1).

Prestalk- and Prespore-Specific Monoclonal Antibodies. By the use of indirect immunofluorescence techniques (as described in the *Materials and Methods*), supernatants of B6 and

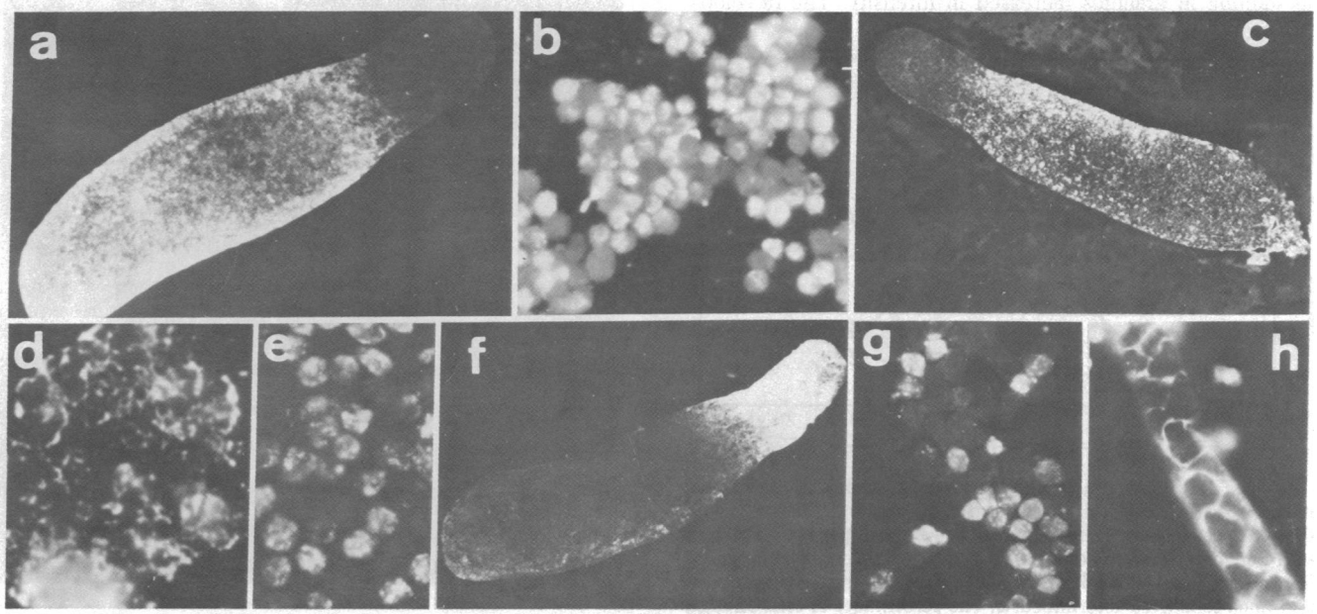

FIG. 1. Photomicrographs of immunofluorescent staining by prespore- and prestalk-specific monoclonal antibodies. Cells and sections were stained by the use of indirect immunofluorescent techniques. (*a*, *c*, and *f*) Sections of migrating slugs. (×130, ×100, and ×100, respectively.) (*b*, *d*, *e*, and *g*) Chemically (*b*, *e*, and *g*) or mechanically (*d*) disaggregated slug cells. (×490.) (*h*) A stalk. (×750.) *a* and *b* were stained with B6 antibody; *c*, *d*, and *e*, with SB5; *f*, *g*, and *h*, with C1.

SB5 clones stain only the posterior prespore region of migrating slugs, showing that they produce prespore-specific antibodies (Fig. 1 *a* and *c*). B6 antibody stains about 70% of disaggregated slug cells in their cytoplasmic granules (Fig. 1*b*), whose size and number are similar to those of PSVs. This antibody stains neither stalk cells nor mature spores (Table 1), unlike polyspecific antispore serum, which stains the surface of spores (4). However, when applied to nascent spores, B6 antibody stained only on their surface. This suggests that the antigen may be secreted along with PSVs at the time of spore formation but rapidly lost thereafter. In contrast to B6, SB5 produces an antibody that stains extracellular as well as intracellular material of prespore cells (Fig. 1*d*). The material tends to be removed even by mechanical disaggregation of slug cells. When chemically disaggregated, slug cells showed no staining on the surface but weak staining in some cytoplasmic granules (Fig. 1*e*), which seems to be the material waiting for excretion. The antibody stains the surface of mature spores but no part of stalk cells.

Supernatant of C1 clone specifically stains the anterior prestalk region of migrating slugs, indicating the presence of a prestalk-specific antibody (Fig. 1*f*). This antibody stains *ca.* 20% of disaggregated slug cells in their cytoplasmic granules (Fig. 1*g*). Stalk and disc cells of mature fruiting bodies are stained on their surfaces (Fig. 1*h*), but no part of spore cells. Because the stalk sheath itself is unstained, the staining of the stalk cells is apparently due to staining of the cytoplasm remaining alongside the walls of stalk cells. Staining characteristics of D4 antibody are the same as those of C1.

Developmental Changes of Prestalk and Prespore Antigens. The appearance of prestalk and prespore antigens during development was examined by the use of the cell type-specific monoclonal antibodies. Cells were disaggregated from cell masses at various stages of development and stained with the antibodies, by using indirect immunofluorescence techniques.

A prespore-specific antibody (B6) stained no cells until 12 hr of starvation when tight aggregates formed. As shown in Fig. 2, stained cells thereafter rapidly increased in number, reaching a level of 70–80% of total cells at the slug stage. The proportion of stained cells thereafter remained constant, although the staining in granules increased in intensity. The developmental kinetics of the cells stained with this antibody coincided well with that of the cells stained with polyspecific antispore serum (Fig. 2).

A prestalk-specific antibody (C1) stained no growth phase cells, but a small fraction (<3%) of cells was stained after 3 hr of starvation. The number of stained cells thereafter increased rapidly to a maximum (*ca.* 80% of total cells) before tight aggregates formed and then fell. Cells showed staining in fine cytoplasmic granules, but there was considerable heterogeneity in the number of stained granules the cells contained (Fig. 3*b*). Cells containing many granules—i.e., strongly stained cells—steadily increased in number during the preaggregation period and reached a level of *ca.* 20% of total cells at the slug stage, the value corresponding to the ratio of prestalk cells. During this period, granules of the cells also increased in both size and staining intensity. In contrast, the number of weakly stained cells decreased during slug formation. This gave rise to clear differential staining between prestalk and prespore cells within a slug (Fig. 1*f*). A similar developmental kinetics of the stained cells was obtained with cells starving in a liquid shake culture (data not shown).

To examine if there is any relationship between the appearance of stained cells and their location, washed vegetative cells were allowed to develop on cover glasses, fixed *in situ*, and stained with the antibody. As shown in Fig. 3 *a* and *b*, stained cells appeared at random and were not morphologically distinguished from unstained cells. Even at a later stage when cells aggregate in streams, strongly and weakly stained as well as unstained cells were randomly distributed (Fig. 3*c*). Essentially

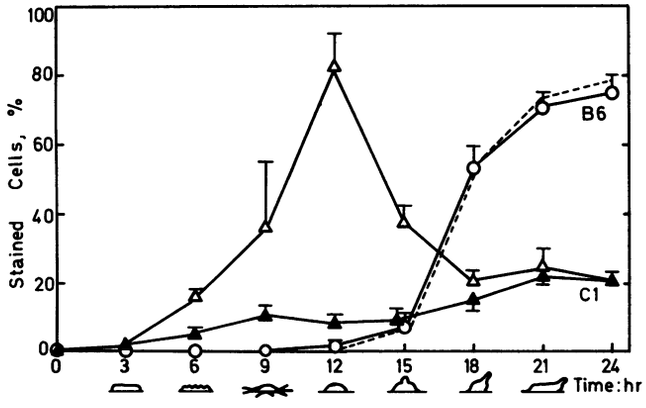

FIG. 2. Developmental changes of cells stained with monoclonal and polyspecific antibodies. Washed exponential growth phase cells were allowed to develop on Millipore filters (5×10^6 cells per cm^2) saturated with Bonner's standard solution. At times, cells were disaggregated, fixed, and stained with B6 (○) or C1 (△) antibodies and the percentages of stained cells were determined. ▲, The percentages of cells strongly stained with C1. The cells were also stained with fluorescein isothiocyanate-conjugated polyspecific antispore serum (---), as described previously (12). The abscissa indicates the time after starvation and developmental stages attained are illustrated below in diagrams. Bars indicate standard deviations.

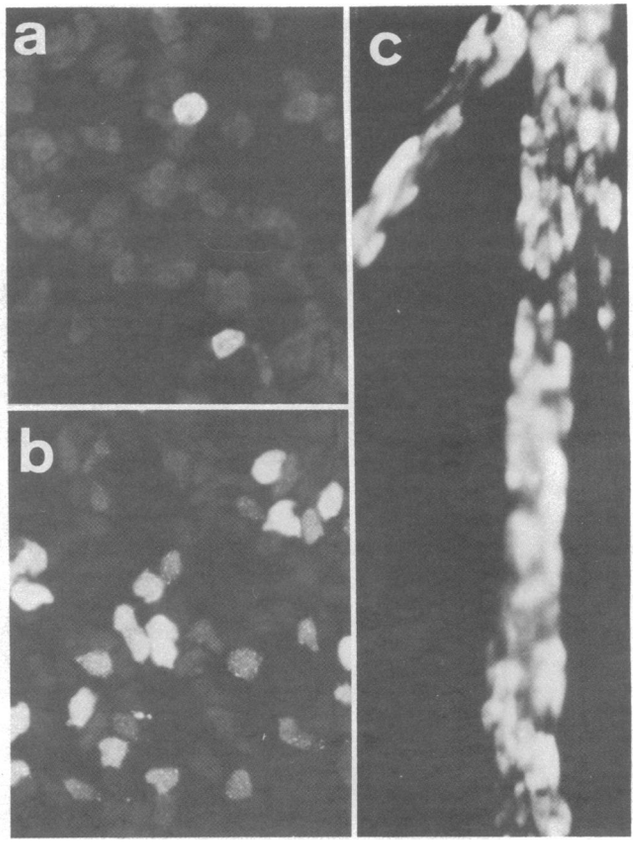

FIG. 3. Photomicrographs of immunofluorescent staining by C1 antibody. Washed growth phase cells were allowed to develop on cover glasses thinly coated with agar (2%), fixed *in situ* after 3 (*a*), 9 (*b*), and 10 (*c*) hr of starvation, and stained with C1 antibody as described in the legend to Fig. 1. (*a* and *b*, ×480; *c*, ×180.)

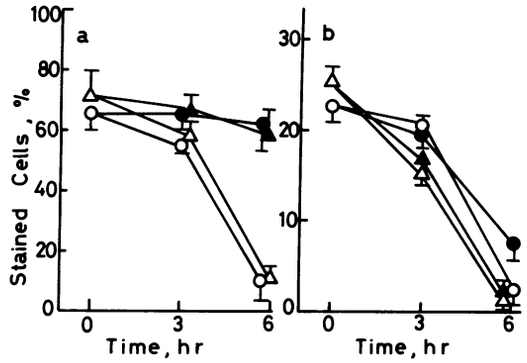

FIG. 4. Disaggregation-induced changes in the antigens. Cells chemically disaggregated from slugs were incubated, and, at times, fixed and stained. The percentages of stained cells were determined. (*a*) Staining with prespore-specific B6 antibody (○, ●) and antispore serum (△, ▲). (*b*) Staining with prestalk-specific C1 (○, ●) and D4 (△, ▲) antibodies. Open and closed symbols indicate incubation of the cells without and with 1 mM cyclic AMP, respectively. The abscissa indicates the time after disaggregation. Bars indicate standard deviations.

the same results as described above were obtained with another prestalk-specific antibody D4 (data not shown).

Disaggregation-Induced Changes in the Antigens. Because many of the products of postaggregative differentiation are known to be lost after disaggregation of slugs (23–25), the effects of disaggregation on prestalk and prespore antigens were examined. As shown in Fig. 4*a*, a prespore antigen reactive against B6 antibody was rapidly lost after disaggregation, but the presence of 1 mM cyclic AMP almost completely prevented the loss. This was also the case with another prespore antigen reactive with SB5 (data not shown). Prestalk antigens reactive with C1 and D4 were also decomposed after disaggregation, but the decomposition was not prevented by exogenous cyclic AMP (Fig. 4*b*).

DISCUSSION

To examine the processes of prestalk and prespore differentiation of *D. discoideum*, we selected cell type-specific monoclonal antibodies from those that are reactive against slug cells but not against vegetative cells. The present study showed that cells stained with two prespore-specific antibodies (B6, SB5) first appear after 12–14 hr of starvation within tipped cell aggregates. This coincides in time with the first appearance of prespore cells as detected by polyspecific antispore serum (12) and of PSV (26). Likewise, prespore-specific mRNAs detected by cDNA clones were recently shown to become detectable around this time (27).

The antigens reactive with B6 and SB5 antibodies were lost after disaggregation of slugs and the loss was prevented by exogenous cyclic AMP. These results also agree well with those obtained with the other aforementioned prespore markers (23, 24, 27).

B6 antibody stains cytoplasmic granules of prespore cells and the antigen is secreted during spore formation and rapidly decomposed thereafter. These results suggest that the antigen is a PSV component other than the acid mucopolysaccharide that is reactive with polyspecific prespore serum (5) and that remains on the spore coat (28). By contrast, SB5 antibody reacts against extracellular material of prespore cells. This appears different from the antigens detectable by prespore-specific monoclonal antibodies obtained by Gregg *et al.* (29), for the latter are localized on the cell surface.

Cells stained with two prestalk-specific monoclonal antibodies (C1, D4) began to appear after 3 hr of starvation, indicating that prestalk antigens become synthesized even before cells begin to aggregate. This contrasts with prespore cells whose antigens first appeared only after a tight cell aggregate formed (12–14 hr). The present result is consistent with the finding of Mehdy *et al.* (27) that the synthesis of prestalk-specific mRNAs becomes detectable after 7.5 hr of starvation and does not require cell–cell contact. After their initial appearance, the number of cells stained with the prestalk antibodies continued to increase and reached a maximum of 80% at 12 hr, the value being much higher than that of prestalk cells within slugs. The fact that this maximum coincides in time with the appearance of prespore cells suggests that all of the cells have a tendency to become prestalk cells first. The above results also indicate that cells that have once undergone prestalk differentiation become converted to prespore cells when the latter begin to differentiate within cell aggregates with tips.

Another feature of the early periods of prestalk differentiation is the considerable heterogeneity: cells varied much in the initiation and the degree of differentiation. The fact that strongly stained cells appeared randomly in the aggregation field suggests that the variation reflects a tendency of cells to become prestalk cells. It is possible that cells having the least tendency are the latest to become prestalk cells during aggregation and the first to become prespore cells within tipped cell aggregates. Differentiated prespore and prestalk cells are then sorted out to produce the pattern within slugs (22).

An alternative interpretation of the above results is also possible: the antigens reactive with C1 and D4 antibodies are synthesized in all of the cells during aggregation and are not related to prestalk cell differentiation. During slug formation, they are selectively lost in prespore cells while they are kept in prestalk cells. However, this interpretation appears inconsistent with the facts that the proportion of cells strongly stained with the antibodies never exceeded that of prestalk cells within slugs (cf. Fig. 2) and the antibodies' staining intensity within these cells continued to increase up to the culmination stage and that the antigens were lost during dedifferentiation of prestalk cells upon disaggregation of slugs (cf. Fig. 4). At any rate, as only a few prestalk markers are available at present, it seems difficult to draw a decisive conclusion on the temporal relationship between prestalk and prespore differentiation; this must wait until a more complete picture of the origin and fate of a range of markers is available.

Both C1 and D4 antibodies stain cytoplasmic granules of prestalk cells. The fact that prestalk cells within slugs contain many autophagic vacuoles suggests that the antibodies are reactive with antigens within the vacuoles. According to Yamamoto *et al.* (30), autophagic vacuoles initially form in all of the cells during aggregation but later are lost in prespore cells. This agrees well with the developmental kinetics of the cells stained by the antibodies (cf. Fig. 2). However, identification of the stained granules must await future electron microscopic studies.

Upon disaggregation, slug cells lost the prestalk antigens regardless of the presence of cyclic AMP, suggesting that the maintenance of the antigens may depend on some factor other than cyclic AMP. This is consistent with the observations that slug disaggregation brings about loss of autophagic vacuoles from prestalk cells (A. Yamamoto, personal communication) but appears curious in view of the fact that the antigen synthesis is initiated before cells form contacts. The reason for this is presently unknown. The failure of cyclic AMP in preventing disaggregation-induced loss of the prestalk antigens may not be common to all prestalk markers, for prestalk-specific mRNAs are synthesized after disaggregation in the presence of cyclic AMP (27).

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