A DEVELOPMENTALLY - CONTROLLED HEMAGGLUTININ FROM THE DIFFERENTIATING PROCARYOTE, *MYXOCOCCUS XANTHUS*

by

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ABSTRACT

During nutrient depletion, the bacterium Myxococcus xanthus undertakes a cycle of development resulting in the formation of fruiting bodies containing myxospores. A hemagglutinin specific for sheep and rabbit erythrocytes was isolated from extracts of developing M. xanthus (wild type strain A^+S^+). The specific activity of the hemagglutinin was shown to peak between 18 - 24 hours after plating on solid starvation medium (agarose-fruiting medium). This time period corresponded to the aggregation stage of the developmental life cycle. Three motility mutants, incapable of aggregating or forming fruiting bodies were also tested for the presence of hemagglutinating activity. Only one mutant, A^+S^-, showed an increase in agglutinin activity, however the peak was 3.5 fold lower than that of wild type cells. The other two motility mutants, A^-S^+ and A^-S^-, failed to show any increase in hemagglutinin activity after plating. Hemagglutinin activity in the wild type strain did not increase in liquid cultures induced to form myxospores with 0.5 M glycerol, nor in liquid modified Al medium. The latter is identical to fruiting medium, except the solidifying agent, agarose, was absent. The agglutinin was most likely a protein, with a molecular weight between 25,000 and 30,000 daltons. Its binding to mono- or disaccharides could not be established. Hemagglutinin inhibition studies indicate it may recognize more complex carbohydrate-containing structures.

The hemagglutinin may function in binding bacteria together during aggregation. Similar proteins (lectins) have been discovered in 6 species of eucaryotic slime molds which undertake a developmental cycle similar to M. xanthus.

In memory of my mother, Lil Stern,

to my family,

and to Allan.

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A_{280}	absorbance at 280 nm
BSA	bovine serum albumin
CM - Sephadex	carboxymethyl - Sephadex
DEAE - cellulose	diethylaminoethyl - cellulose
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease I
EDTA	ethylenediamine tetraacetate
EGTA	ethyleneglycol-bis-N,N'-tetraacetic acid
mRNA	messenger RNA
PBS	phosphate buffered saline
RBC	red blood cell
RCF	relative centrifugal force
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TE buffer	Tris - EGTA buffer
TRIS	tris (hydroxymethyl) aminomethane

A Developmentally - Controlled Hemagglutinin from the Differentiating Procaryote, *Myxococcus xanthus*

INTRODUCTION

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Cell-cell interactions, particularly cellular adhesion, have been the subject of many studies. According to Grinnel (1978), adhesiveness is a fundamental cell property that plays a role in developmental processes such as migration of cells during embryogenesis and morphogenesis; in tissue and organ stability, inflammation, the behaviour of malignant cells, and in disorders of platelet and leukocyte function. The complexity of cellular associations in these systems suggests a high level of development of cellular recognition processes. To understand the mechanism of recognition in these complex systems, isolation of the specific molecules that mediate cellular recognition is necessary. This may be accomplished more readily in simpler systems, and, assuming that the general mechanisms are conserved in some form, extrapolation may be made to more complex systems.

The myxobacteria are procaryotes that undergo a developmental life cycle, during which they aggregate on solid medium to form multicellular fruiting bodies. Because of this unique developmental cycle, they offer an excellent opportunity to investigate the mechanism of cell interactions in a simple system. The life cycle of the myxobacteria resembles closely that of the eucaryotic slime mold amoebae. Studies of six species of slime molds have shown that a carbohydrate-binding hemagglutinin may mediate cellular adhesion during the aggregation stage of development. Because of their similar developmental life cycles, this investigation was undertaken to determine whether the myxobacteria, Myxococcus xanthus also elaborates a carbo-

hydrate - binding hemagglutinin during its developmental life cycle.

HISTORICAL

HISTORICAL

Myxobacteria (order Myxobacterales) are ubiquitous in nature, occurring in well aerated soil, and decomposing plant and animal material (McCurdy, 1974; Dworkin, 1966). They were first described by Roland Thaxter in 1892 in a paper published in the Botanical Gazette as "...bright orange-colored growths occurring upon dead wood, fungi and similar substances..." (Thaxter, 1892). The order is composed of several genera of gram negative, rod-shaped bacteria that under certain conditions form myxospores contained within fruiting bodies. The order is subdivided into four families based on morphologies of vegetative cells, myxospores, and fruiting bodies. The four families are Myxococcaceae, Archangiaceae, Cystobacteraceae, and Polyangiaceae (McCurdy, 1974). They are further characterized by slow, gliding motility also displayed by several unrelated procaryotic groups, and not well understood in any of them. Gliding occurs only at interfaces (usually solid-liquid) and involves the slow, smooth movement of rod-shaped cells, embedded in a layer of slime, in the direction of their long axis (McCurdy, 1974). A third characteristic is their ability to hydrolize insoluble macromolecules (Dworkin, 1966).

The myxobacteria are unique among procaryotes in their developmental cycle. When nutrients are amply supplied, vegetative cells reproduce by binary transverse fission. However, under conditions of nutrient deprivation, the cells aggregate by gliding on solid surfaces to form multicellular fruiting bodies, inside which the vegetative cells are transformed into resting myxospores (Dworkin, 1966 & 1973). Myxospores may be spherical, ellipsoidal or rod-shaped, and are resistant to heat, sonication and dessication.

The myxobacteria produce fruiting bodies of varying complexity. Some genera, such as Chondromyces and Stigmatella, form elaborate fruiting structures consisting of stalks bearing sporangia containing the resting myxospores (McCurdy, 1974). The stalks are composed of hardened slime within which are embedded a few cells (Reichenbach & Dworkin, 1969). Other genera, such as Myxococcus, exhibite simple fruiting structures consisting only of a mound of cells embedded within a polysaccharide slime (McCurdy, 1974; Wireman & Dworkin, 1975). The myxospores of the genus Myxococcus are optically refractile, and are about 2 µm in diameter (Sudo & Dworkin, 1968). Under appropriate conditions, the spores germinate to release vegetative cells.

Myxococcus xanthus, a well-studied species of myxobacteria, are long slender rods, with tapered or rounded ends, measuring 0.5 - 1.0 by 4.0 - 10.0 µm. They are strict aerobes, several strains of which are capable of growth in a dispersed state in liquid culture (McCurdy, 1974; Wireman & Dworkin, 1975). In nature, M. xanthus secretes bacteriolytic enzymes and several proteases that permit it to prey on bacteria (Sudo & Dworkin, 1972). A defined medium containing 17 amino acids and various salts can be used to culture M. xanthus (Dworkin, 1962). No stimulation of growth was found with a variety of organic compounds including several sugars, sodium acetate, sodium citrate, guanine and uracil. Only glycogen was found to produce a marked stimulation, although its role was unclear. Its function is not solely a physical one, since it disappears from the medium, however, since the growth of the cells is not appreciably stimulated by glucose, or by sugars in general, it is unlikely that the function of glycogen is to provide an external source of sugar (Dworkin, 1962). A minimal medium containing only seven amino

acids, salts and vitamin B-12, with pyruvate and aspartate as carbon sources was recently constructed (Bretscher & Kaiser, 1978). The amino acids, isoleucine, leucine, valine and methionine were essential amino acids - virtually no growth appears in their absence. Other amino acids, while not essential, stimulated growth (i.e. phenylalanine, asparagine, and spermidine). The generation time of M. xanthus in this minimal medium was between 22 and 30 hours at 29°C (Bretscher & Kaiser, 1978), whereas in complex medium (e.g., 1% Difco Casitone and $MgSO_4$), the generation time was 200 to 250 minutes (Wireman & Dworkin, 1975). The amino acid, phenylalanine, while not required for vegetative growth of most strains in liquid culture, was required for vegetative growth on solid medium. The development of a defined minimal medium was a necessary prerequisite for investigating the developmental process of fruiting body formation in Myxococcus.

The formation of fruiting bodies in M. xanthus is subject to multiple controls. Many studies have been done on the nutritional control of fruiting body formation, and although there is a fair amount of information in this field, the precise mechanism is not well understood. The stimulus for fruiting body formation is nutrient deprivation - more specifically, the absence of certain specific amino acids (Dworkin, 1963). Hemphilll and Zahler (1968) determined that fruiting occurred when any amino acid required for normal growth became limiting in the environment. High concentrations (10mM) of phenylalanine, tryptophan, and methionine prevented fruiting without affecting normal vegetative growth (Hemphill & Zahler, 1968; Witkin & Rosenberg, 1970). The three amino acids, when present at high concentrations, suppressed fruiting under conditions where it would otherwise have occurred. The conclusion

drawn was that induction of fruiting was controlled by two factors - the disappearance of critical elements of nutrition, and the suppression of fruiting by the three specific amino acids (Hemphill & Zahler, 1968).

A second level of regulation in fruiting involves cyclic AMP and ADP. Campos and Zusman (1975) observed that adenine nucleotides, especially cAMP and ADP, stimulated fruiting in agar containing low levels of nutrients, sufficient for vegetative growth but not fruiting. When nutrient levels were further decreased to allow fruiting, the presence of the nucleotides vastly increased the number of fruiting bodies formed.

In addition to the above, fruiting body formation is also dependent upon the initial density of the cells plated (Wireman & Dworkin, 1975). A threshold level of 5.0×10^8 cells/ml was necessary for fruiting, although there were sufficient cells at lower concentrations to form fruits.

With the initiation of fruiting, the long thin vegetative cells migrate in an orderly fashion towards aggregation centers, where macroscopic fruiting bodies are formed. Inside the fruiting body, the vegetative cells round up to form ellipsoidal, then spherical myxospores (Wireman & Dworkin, 1975). Depending upon conditions such as nutrition, cell density, etc., fruiting can take 48 - 72 hours. During fruiting body formation by wild-type cells of M. xanthus, massive cell death occurs. About 60 - 80% of the original vegetative cell population lyse, and only about 20% survive as myxospores in the completed fruiting bodies. The role of this regulated cell death is not known (Wireman & Dworkin, 1975).

Myxospore formation can take place in the absence of fruiting body formation. Vegetative cells can be induced to form myxospores in liquid culture by the addition of glycerol (final concentration 0.5M) or other compounds with primary or secondary alcohol groups (Sadler & Dworkin, 1966).

The myxospores formed by glycerol induction resemble those formed within fruiting bodies in terms of gross morphology and resistance properties (Kottel et al., 1975). The entire conversion, from long thin vegetative cells to round, optically refractile, resistant resting cells requires about 120 minutes, and is relatively synchronous. Unlike myxospore formation in fruiting bodies where there is massive cell death, during glycerol induced conversion there is no decrease in cell numbers (Gibson & Dworkin, 1964; Sadler & Dworkin, 1966). Myxospore formation induced by glycerol, and that within fruiting bodies are genetically separate events, even though the resulting myxospores are similar. Most glycerol non-inducible mutants of M. xanthus are capable of forming fruiting bodies containing myxospores (Parish et al., 1976).

The cell wall of vegetative cells and myxospores contain a peptidoglycan similar in composition to that found in other eubacteria. Myxospores contain approximately the same amount of peptidoglycan as vegetative cells, however, its structure is altered as the cells undergo transformation from rods to spheres. The peptidoglycan in the vegetative cells exists as separate patches or plates separated by non-peptidoglycan material, whereas the myxospore peptidoglycan is a continuously cross-linked sheath (White et al., 1968). Johnson et al. (1972) showed that part of the process of restructuring the cell wall during myxospore formation involves cross-linking between peptides of the peptidoglycan, or between peptidoglycan peptides and other cell wall components.

Electron microscopy of glycerol-induced myxospores as well as fruiting body myxospores revealed the presence of an extracellular envelope termed the myxospore capsule or coat (Kottel et al., 1975) which is distinct from the surrounding polysaccharide slime of vegetative cells

and myxospores. White et al. (1968) had previously found that galactosamine and glycine rapidly accumulated in cell walls when the cells were converting from ovoids to spheres, and this was believed to reflect the formation of this coat material.

The extracellular coat was isolated and characterized by Kottel et al., (1975) by transmission and scanning electron microscopy. The dry weight composition was as follows: 75% polysaccharide, composed of galactosamine and glucose only; 14% protein; 8% glycine; and <1% organic phosphorous. The coat is a compact structure approximately 25 to 50 nm thick, of one or possibly more layers. Sections of fruiting spores from week-old fruits revealed a thicker coat of several layers. However, their compositions were similar.

Sutherland and MacKenzie (1977) determined that the glucose of myxospore coats was present as an α - 1,3 - linked glucan polymer, whereas the galactosamine was a component of a different polymer. In M. xanthus the glucan was postulated to be bound to the peptidoglycan. This α - 1,3 - glucan was common to the microcyst walls of several other species of procaryotes as well as at least one species of eucaryote (Zonneveld, 1971). Lectin agglutinability studies of the myxospores showed that the glucan is a surface component that is readily accessible to the lectin. In contrast, the galactosamine portion was not readily agglutinable - perhaps because other material occludes the galactosamine - containing polymer. A correlation was found between the incorporation of galactosamine into the cyst wall and the development of resistance to sonication.

Metabolic studies have been done on the changes in enzyme activity associated with conversion to myxospores in glycerol-induced systems. With the onset of sporulation, a number of enzymes are induced, including

those of the glyoxylate pathway (Dworkin, 1972), and the seven enzymes required to synthesize uridine 5' - diphosphate (UDP) N-acetyl galactosamine (UDP GalNAc), the major precursor in coat synthesis, from fructose 1,6 - diphosphate (Filer et al.1977a). The specific activities of these enzymes increased 4.5 - 7.5 fold after 2 hours of induction. The increase in specific activity of these enzymes was not observed in a mutant unable to be induced by glycerol. Studies on the incorporation of acetate and glycine by glycerol - induced myxospores provided circumstantial evidence that a phospholipid intermediate may be involved in the biosynthesis of the M. xanthus coat polymer (Filer et al., 1977b).

The life cycle of the procaryotic myxobacteria resembles closely the developmental life cycle found in an unrelated eucaryotic organism - the slime molds. The slime molds maintain a normal vegetative cycle during which they feed on bacteria and live independently. Like the myxobacteria, however, under conditions of nutrient deprivation, the amoebae aggregate into multicellular pseudoplasmodia containing up to 10^5 cells each (Barondes & Rosen, 1976). In the next 12 hours, the pseudoplasmodia undergo further differentiation to form a multicellular fruiting body consisting of a stalk and spore cap containing resistant, resting spores. The spores germinate under favorable environmental conditions to become vegetative amoebae (Barondes & Rosen, 1976). Because of its unique life cycle, much research has been done on determining the mechanism of aggregation and fruiting body formation in the slime molds, particularly with the genus Dictyostelium discoideum.

Aggregation and fruiting body formation in the slime molds is controlled at several levels. The stimulus that brings about aggregation and fruiting body formation is starvation, which induces some cells to

produce slow, rhythmic pulses of the attractant cyclic AMP (Bonner et al., 1972). The remaining cells produce cyclic AMP receptors on their cell surfaces that enable them to bind the pulsed signal. The amoebae respond in two ways to these signals. First, they move in the general direction of the signal source, and second, about 12 seconds after receiving the signal, the amoebae themselves emit a pulse of cAMP. The amoebae close to the center are prevented from relaying the signal inwards by becoming refractory to further stimulation for several minutes after producing a pulse. In this way, a series of waves of cAMP production, destruction, and response, move outward from the centers as the amoebae move inwards (Newell, 1977). Not all slime molds produce cyclic AMP as the chemotactic agent, but most produce some kind of acrasin (chemotactic substance). The discovery of cyclic AMP in Dictyostelium led to the investigation and discovery of cyclic AMP and ADP as stimulatory agents in Myxococcus (Newell, 1977).

The second phase of aggregation involving cell recognition and specific cell adhesion depends upon the synthesis of specific cell surface components - contact sites A and slime mold lectins (Newell, 1977; Barondes & Rosen, 1976). Univalent antibody fragments specific for contact sites A were bound to the cell surface only when the cells were in the aggregation - competent state (Huesgen & Gerisch, 1975). The contact sites have been partially purified and are a minor cell surface glycoprotein in aggregating cells, present at about 2×10^5 copies per cell. Univalent antibody fragments render the cells unable to form the specific adhesion characteristic of aggregating cells (Beug et al., 1970). Müller and Gerisch (1978) determined that antibody fragments specifically block end to end association of aggregating cells. In addition to cell aggregation mediated by contact sites, cell surface adhesion is also mediated

by slime mold agglutinins or lectins (Rosen et al., 1973). Lectins are proteins or glycoproteins that bind non-covalently to specific carbohydrate groups on the surfaces of cells (Brown & Hunt, 1978). They can be detected by their ability to bind and agglutinate red blood cells (rbc's). Hemagglutinating activity was first discovered by Stillmark in 1888 (Boyd, 1970) in extracts of beans of castor and abrus plants. Because of their erythrocyte - binding activity, they were named hemagglutinins, and more specifically phytohemagglutinins, because they were isolated form plant sources. Since then, hemagglutinins have been found in extracts of bacteria, cellular slime molds, invertebrates such as snails and the horseshoe crab, lower vertebrates such as fish, and over 800 species of plants (Sharon & Lis, 1972). Because they agglutinate cells other than erythrocytes, and because they have been isolated from a variety of sources, the term "lectin" was used to replace phytohemagglutinin. In addition to their ability to agglutinate erythrocytes, lectins exhibit a variety of other interesting biological and chemical properties involving cell surface interactions.

The presence of lectins in differentiating slime mold amoebae was first discovered by Rosen et al., (1973), who found that a protein in crude extracts of Dictyostelium discoideum caused the agglutination of sheep red blood cells. The protein, called discoidin, increased in activity over 400 fold during aggregation, when cells developed cohesiveness. Studies showed that the agglutination activity of the lectin could be blocked by the sugar N-acetyl-D-galactosamine. Several other monosaccharides, including D-galactose, also inhibited hemagglutination, but were less effective, and most were totally inactive (Barondes & Rosem ,1976; Rosen et al., 1974).