Effects of Glucosamine on Lysis, Glycerol Formation, and Sporulation in *Myxococcus xanthus*

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Glucosamine (GlcN), which has previously been shown to rescue fruiting body formation, lysis, and sporulation in a developmental mutant (G. Janssen and M. Dworkin, *Dev. Biol.* 112:194-202, 1985), induced lysis in vegetative and developing wild-type cells and inhibited fruiting body formation. It also resulted in a transient, intracellular increase in the concentration of glycerol, a known sporulation inducer, and sporulation of the surviving cells. Phospholipase activity, which was shown to be normally developmentally regulated, increased 7.6-fold after treatment of vegetative cells with 50 mM GlcN. Likewise, autocidal activity, which normally increased 18 to 24 h after the initiation of development, increased 20% when vegetative or developing cells were exposed to GlcN. Two mutants resistant to GlcN-induced lysis (MD1021 and MD1022) were isolated and showed neither an increase in autocide production nor an increase in phospholipase activity in response to added GlcN. MD1021 was developmentally deficient, and GlcN rescued fruiting body formation as well as phospholipase activity and autocide production. We propose that GlcN exerts its lytic effect by regulating the activity of phospholipase enzymes that release autocides, compounds that are believed to be responsible for developmental autolysis. GlcN-induced sporulation was found to depend on several factors: the initial cell density, the amount of lysis induced by GlcN, and the presence of tan-phase variants. An initial cell density of greater than 2×10^5 cells per ml was required to support GlcN-induced sporulation, and sporulation did not occur unless 50 to 75% of these cells had lysed. Mutants that were resistant to GlcN-induced lysis also did not sporulate in the presence of GlcN. The effects of GlcN on developing cells depended on the concentration of GlcN added; the addition of low concentrations of GlcN resulted in enhancement of sporulation, while higher concentrations resulted in the inhibition of sporulation. The ultrastructure of GlcN-induced spores resembled that of spores induced by the exogenous addition of glycerol, in contrast to spores isolated from mature fruiting bodies. A model by which GlcN may regulate both lysis and sporulation is presented.

Myxococcus xanthus is a prokaryote that is distinguished by its ability to carry out a complex developmental life cycle. In response to starvation, cells glide into aggregation centers and mature into spore-filled fruiting bodies. During the process that leads to sporulation, a portion of the cells have been observed to undergo lysis (23, 48, 49). Sporulation occurs as the culmination of a developmental process within a maturing fruiting body (35). The formation of fruiting bodies depends on three requirements being met: the presence of a solid surface, a high cell density (48), and depleted nutrient supply (6, 16). Spores formed within the mature fruiting body are metabolically quiescent (18) and resistant to heat, desiccation, UV light, and breakage by sonic oscillation (44).

Intercellular coordination of fruiting body formation has been shown to require the exchange of regulatory signals (20, 29, 30). Hagen et al. (20) described a set of conditional nonsporulating mutants that were divided into four groups (A, B, C, and D) which undergo extracellular, phenotypic complementation. These mutants are believed to be deficient in the exchange of particular signals that are required for development. Several of the group C mutants were found to be deficient in lysis, sporulation, and fruiting body formation (23, 42). Janssen and Dworkin identified and partially purified a cell-associated factor, called DS, for development-stimulating activity, which phenotypically complemented lysis, sporulation, and fruiting body formation in the group C mutant (23). The effect of DS activity on the group C mutant

could be replaced by adding the amino sugar glucosamine (GlcN) or mannosamine (ManN). GlcN is thought not to correct the genetic lesion directly (23, 26, 40) but rather to bypass the mutation and reinitiate the lysis and sporulation pathway at a point further down from the mutational block (40). It has been proposed (23) that GlcN is, or mimics, the signal that in wild-type cells initiates developmental lysis and sporulation.

During the course of an attempt to understand the mechanism of the GlcN rescue of the group C mutants, we found that GlcN, when added to the developmentally proficient parent, induced premature autolysis, glycerol formation, and myxosporulation.

Wireman and Dworkin originally found that sporulation was accompanied by a significant amount of cell lysis (23, 48, 49). Subsequently, lysis has been used as a marker for development (35, 41, 42) and has been assumed to be a natural part of the developmental process. This has been based on a close correlation between development and autolysis; however, no clear evidence has been presented to formally establish a cause and effect relationship. O'Connor and Zusman (33) have presented evidence that, under certain conditions, autolysis is less extensive than has been reported and have suggested that it is not an integral part of development but rather is induced by the experimental mishandling of fragile developing cells. On the other hand, Rosenbluh et al. (36), using an elegant technique whereby cells were trapped in agarose microbeads and then induced to develop under conditions that essentially eliminated manipulation of the developing cells, showed extensive lysis occurring concomitantly with the appearance of myxospores.

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It has been proposed that the lysis seen during development was due to the action of myxobacterial autocides (46). The autocides are a group of five different compounds that lyse vegetative and resting cells of the producing and closely related strains (46). Autocide 1 (AM1) is responsible for the major autolytic activity; it has been purified and identified as a pool of free fatty acids (47). AM1 has been shown to rescue sporulation in a major class of sporulation mutants called *dsg* mutants. The exact developmental role of AM1 is not known, but Rosenbluh and Rosenberg have proposed that it is involved in altering membrane permeability early in development and, as a result, is involved in autolysis later in development (39). AMV has also been purified and was identified as phosphatidylethanolamine (19), the major phospholipid in the *M. xanthus* inner and outer membrane (34). The lytic activity of AMV was found to be associated with the fatty acid moiety of the phospholipid, and its autocidal activity was thought to be due to the release of AM1 by the action of one or more phospholipases (19).

It is possible to bypass the multicellular developmental process altogether and chemically induce vegetative cells to sporulate (17). Glycerol (0.5 M) is normally used as the chemical inducer, but other secondary alcohols, such as dimethyl sulfoxide, will also induce sporulation (17, 27). Chemical induction of sporulation by glycerol is rapid, occurring within 120 min, and is also synchronous and relatively complete (95%) (17). The mechanism of glycerol induction is not known. It is thought to activate directly the steps, normally occurring during fruiting body formation, which lead to changes in cell shape and the acquisition of resistance properties (25). The question of whether glycerol plays a role in normal developmental sporulation has remained unanswered. It was of additional interest to discover that GlcN-induced lysis was also accompanied by a transient accumulation of glycerol and subsequent sporulation.

It has been our working hypothesis that GlcN initiates a cascade of events that results in sporulation and lysis. We have shown that GlcN induces lysis and sporulation in vegetative cells. We also demonstrated that GlcN affects the activity of phospholipase enzymes and the release of the autocide AM1 in vegetative and developing cells. Furthermore, we have shown that increased phospholipase activity and AM1 production are correlated with development. We also report that the addition of GlcN causes the transient, intracellular accumulation of the developmental inducer glycerol, and presumably, as a consequence, induces the formation of myxospores. Finally, we present a model that incorporates these events into a developmental pathway that leads to two developmental fates, lysis and sporulation.

MATERIALS AND METHODS

Strains and growth conditions. *M. xanthus* DK1622 (24) was used as the wild-type strain. Strains MD1021 and MD1022 are mutants that are resistant to GlcN-induced lysis. Cells were vegetatively cultured in Casitone (CTT) growth medium containing 1% (wt/vol) Casitone (Difco), 1 mM potassium phosphate (pH 7.6), 8.2 mM magnesium sulfate, and 10 mM Tris-HCl (pH 7.6). Cells were grown at 30°C with shaking. Vegetative growth on solid medium was on CTT agar (CTT growth medium solidified with 1.5% [wt/vol] Bacto Agar [Difco]). Strains MD1021 and MD1022 were grown in CTT medium supplemented with 50 mM GlcN (Sigma Chemical Co., St. Louis, Mo.). The GlcN stock solution (1 M, pH 7.6) was made fresh daily, filter sterilized, and added to sterile CTT medium. Development was in-

duced on TPM agar containing 10 mM Tris-HCl (pH 7.6), 1 mM potassium phosphate, and 8.3 mM magnesium sulfate and solidified with 1.5% Bacto Agar. To induce development, vegetative cells were harvested by centrifugation, washed once in TPM buffer, and resuspended to a density of 8×10^9 cells per ml. The cells were inoculated onto TPM plates (2×10^8 cells per 5- μ l drop, 20 drops per plate) and allowed to dry into the agar. When indicated, TPM agar was supplemented with filter-sterilized GlcN. The plates were incubated in a humid incubator at 30°C.

Isolation of GlcN-resistant mutants. DK1622 was grown to 1×10^8 to 2×10^8 cells per ml, and 1 ml of cells was transferred to a 15-ml tube. Fifteen microliters of EMS (ethyl methanesulfonate) (Sigma) was added to the cells, and the tube was incubated for 2 h at 32°C with frequent, intermittent shaking. CT growth medium (2% Casitone [wt/vol], 1 mM potassium phosphate [pH 7.6], and 8.2 mM magnesium sulfate) was added to the tube, and the cells were harvested by centrifugation. The cells were resuspended in CT medium, and 0.2-ml samples were transferred to small glass tubes. The tubes were slowly shaken overnight in a humid chamber at 32°C. The cells were then plated on CT agar supplemented with 50 mM GlcN. MX4 *hrm*(Ts) (9) was grown on the GlcN-resistant strains, and the lysates were used to transduce DK1622 cells to GlcN resistance. GlcN-resistant colonies were restreaked three times from isolated colonies.

Measurement of cell numbers and viable spore counts. Cell numbers were determined in a Petroff-Hausser counting chamber. The number of heat-resistant, sonication-resistant, viable myxospores was determined by heating a harvested cell suspension to 51°C for 2 h and then sonicating (two 30-s pulses at a 65-W power setting with intermittent cooling) and plating for CFU on CTT agar. The combined heat and sonication treatments resulted in less than 10^{-9} survival for vegetative cells, while not significantly affecting mature myxospore viability.

Assay for GlcN-mediated lysis. Vegetative cells were harvested in mid-logarithmic phase (1×10^8 to 3×10^8 cells per ml), washed once, and resuspended to a density of 2×10^8 cells per ml in CTT growth medium supplemented with GlcN or in morpholinepropanesulfonic acid (MOPS) buffer supplemented with GlcN. Cultures were incubated with shaking at 32°C. Samples were removed, and cells were counted in a Petroff-Hausser counting chamber.

Assay for lytic activity of AM1. AM1 was isolated by using disposable prepacked silica gel (500 mg) mini-columns (Supelclean; Supelco) by the method of Hamilton and Comai (21). Vegetative or developing cells were harvested and lipids were extracted by the method of Bligh and Dyer (4). The organic phase of the Bligh-Dyer extract was dried under nitrogen, redissolved in 2.0 ml of hexane-MTBE (methyl tertiary butyl ether) (200:3), and applied to the column. The vessel containing the sample was washed once with 2 ml of hexane-MTBE (200:3), and the solvent was added to the column. Neutral lipids were eluted with hexane-MTBE (200:3, 10 ml). The column was acidified with 12 ml of hexane-acetic acid (100:0.2), and this fraction was discarded. Fatty acids were eluted with 12 ml of hexane-MTBE-acetic acid (100:2.0:0.2), with a 97.3% recovery as determined by recovery of added [3 H]oleic acid. Phospholipids remained bound to the column. The purity of the eluted free fatty acids was checked by thin-layer chromatography analysis of the eluant. Samples of the eluant were spotted onto silica GF plates (Analtech) and run in ethyl acetate or in chloroform-methanol (7:3). The fraction containing free fatty acids was

dried under nitrogen, resuspended in 100 μ l of MBTE, and assayed for AM1 lytic activity by the filter disk method described in Varon et al. (46). One unit of AM1 activity is defined as that amount which gives a net 1-mm-diameter ring of lysis.

Phospholipase assay. Phospholipase A and lysophospholipase activity were assayed together by a modification of the method of Orndorff and Dworkin (34). This method assays the conversion of phospholipids labeled with [14 C]glycerol to water-soluble products. Vegetative or developing cells were harvested without washing, resuspended in MOPS- Ca^{2+} buffer (10 mM MOPS [pH 7.6], 4 mM magnesium sulfate, 2 mM calcium chloride), and osmotically lysed. The crude lysate was used as the source of phospholipase enzyme. Protein determinations were made by using a Bio-Rad assay (5).

The phospholipid substrate for the reaction was obtained by labeling vegetative cultures of DK1622 in CTT medium containing 0.20 μ Ci of [14 C]glycerol per ml (16.8 μ Ci/mmol) for 24 h. Phospholipids were extracted by the method described by Orndorff and Dworkin (34). Total lipid phosphorus was measured by the method of Chen et al. (11). Assay mixtures contained 80 nmol of crude phospholipid (2.9×10^5 cpm/nmol), 30 μ l of methanol, 90 μ l of Triton X-100, 300 μ l of Tris-acetate (pH 7.6), 75 mM CaCl_2 , and 200 μ g of crude lysate protein. The reaction was carried out at 30°C. After 15 min of incubation, 150- μ l samples were removed and the reaction was terminated by transfer to 30 ml of cold chloroform-methanol (2:1, vol/vol). NaCl (0.5 ml of a 0.3% solution) was added to facilitate solvent partitioning. The mixture was briefly vortexed and then was briefly centrifuged ($2,000 \times g$ for 1 min). Part of the aqueous phase (0.8 ml) was transferred to a scintillation vial containing Ecolume (ICN Biochemicals) scintillation fluid and counted in a scintillation counter. Phospholipase activity is expressed as nanomoles of lipid phosphorus converted to water-extractable form per minute per milligram of protein.

Induction of sporulation. (i) **Glycerol induction.** Glycerol was added to an exponentially growing culture in CTT medium to a final concentration of 0.5 M, and the culture was incubated with shaking for 18 h at 32°C.

(ii) **GlcN induction.** GlcN (10 to 50 mM) was added to an exponentially growing culture, which was then incubated with shaking at 32°C for 24 h.

(iii) **Spores derived from fruiting bodies.** Vegetative cells were harvested, washed in TPM buffer, and resuspended to a density of 2×10^9 cells per ml in TPM buffer. Twenty five-microliter drops (20 drops per plate) were placed on TPM agar plates, and the cells were allowed to develop at 32°C for 96 h in a humid incubator. Fruiting bodies were harvested by scraping the plate with a razor blade and then resuspended in buffer.

Glycerol measurement. Vegetative cells at a density of 2×10^8 cells per ml were incubated with 50 mM GlcN for 3 h, with samples removed for glycerol determination at the indicated times. Cells (2×10^9) were removed by centrifugation and extracted with ethanol for 15 min. The extract was filtered through a glass fiber filter to remove insoluble material, and the filtrate was dried under a stream of nitrogen. The sample was resuspended in 200 μ l of distilled water and centrifuged at $13,000 \times g$ for 15 min in the cold to remove insoluble material. The supernatant solution was stored at -20°C. Glycerol was measured by high-pressure liquid chromatography (HPLC) using an IC-PAK ion-exclusion column (7.8 mm by 30 cm). The column was run at a rate of 1.4 ml/min at ambient temperature, using an isocratic

FIG. 1. GlcN induces lysis in vegetative cells. Vegetative yellow- or tan-colony-phase variant cells (2×10^8 cells per ml) were grown in the presence of various amounts of GlcN for 6 h. Samples were taken at the indicated times and counted in a Petroff-Hausser counting chamber. The values represent the averages of three independent determinations.

0.05% phosphoric acid solvent. The column was standardized with 30 μ l of a 9.95 mM solution of glycerol, eluted at 6.1 min. Blank solvent runs were done between samples to ensure that there was no carryover from one sample to the next.

Electron microscopy of spores. Fruiting body, glycerol, and GlcN-induced spores were harvested on sucrose step gradients (22). Purified spores were fixed with 2.5% glutaraldehyde (wt/vol) in 0.1 M sodium cacodylate (pH 7.6) and 0.05% CaCl_2 (wt/vol) for 4 h at 4°C. The fixative was changed at 2 h. Cells were postfixed in aqueous 1% osmium tetroxide for 1 h at 4°C, washed three times in buffer, and poststained for 2 h in buffered 0.5% uranyl acetate. Dehydration was accomplished by a graded series of ethanol. The spores were embedded in Spurr resin for 3 days and stained for 10 min in ethanolic uranyl acetate and for 1 min in lead citrate. All thin sections were examined with a JEOL 100-CX TEMSAN transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS

GlcN induces lysis in vegetative cells. GlcN has been shown to rescue developmental lysis, sporulation, and fruiting body formation in several developmental mutants (23). In the present study, GlcN was added to vegetative cells and was found to induce cell lysis. The extent of lysis depended on several variables, viz., the concentration of GlcN added, the initial density of cells, and the relative proportions of yellow- and tan-phase variants in the initial vegetative population. The initial cell density was always kept at 2×10^8 cells per ml unless otherwise indicated. At concentrations of 30 to 50 mM GlcN, all cells lysed within 3 to 4 h after the addition of GlcN (Fig. 1). Microscopic examination of vegetative cultures treated for 4 h with 50 mM GlcN showed spheroplast formation and disintegrating cells. No growth of surviving

FIG. 2. Fruiting body formation in the presence of GlcN. Vegetative cultures were harvested, washed once, and resuspended in TPM buffer to a density of 10^{10} cells per ml. A 0.1-ml portion was spread evenly on a TPM agar plate supplemented with various amounts of GlcN, and the cells were allowed to develop in a humid incubator at 32°C. Photographs were taken after a 96-h development.

cells occurred even after several days of subsequent incubation.

Within the range of 10 to 20 mM GlcN, the response was variable (Fig. 1). At the lower concentrations of GlcN, the extent of lysis was highly variable from day to day. This was found to depend on the relative concentrations of yellow- and tan-colony-phase variants (7) in the original vegetative population. Although little is known about the mechanism of phase variation and the function of the different cell types, it may be that the two colony types play different roles during development (40). For example, in order for GlcN to rescue the group C mutant DK731, a certain proportion of tan cells had to be present in the population (23). Our results showed that yellow- and tan-phase variants responded differently to the addition of GlcN in that the tan variants were more resistant to GlcN-mediated lysis. Three hours after the addition of 15 mM GlcN, the population of the yellow variant decreased from 2×10^8 cells per ml to 3×10^7 cells per ml (85% lysis), while the tan variant population only decreased to 1.2×10^8 cells per ml (40% lysis) (32). Lysis could also be induced by the amino sugar ManN (which will also substitute for GlcN in the rescue of the group C mutant DK731 [23]). No other carbohydrate, among the many that were tested, was found to induce lysis (32).

Isolation of mutants resistant to GlcN-induced lysis. In order to understand the mechanism of GlcN-induced lysis, mutants were obtained which would not lyse in CTT growth medium supplemented with 50 mM GlcN. The mutants were generated by using EMS as a mutagen. Two strains were isolated and studied. By using MX4 virus *hrm*(Ts), a lysate was prepared on each strain and used to transduce DK1622

to resistance to GlcN-induced lysis. For MD1021, 26 transductants were isolated, and all were shown to be GlcN resistant and deficient in fruiting body formation (Fig. 2). At concentrations of GlcN greater than 20 mM, the severity of the inhibition of development increased. The two GlcN-resistant mutants, MD1021 and MD1022, had different developmental responses to GlcN. MD1021 was unable to aggregate and form fruiting bodies, but its development was rescued by exogenous GlcN (Fig. 2). In this respect, MD1021 behaved like the group C mutant DK741 (23). As in GlcN-induced lysis of vegetative cells, inhibition of fruiting body formation was a specific event mediated only by amino sugars. GlcN and ManN were the only carbohydrates tested which inhibited fruiting body formation, induced lysis, and rescued the mutants MD1021 and DK741 (32). MD1022 was developmentally proficient in the presence and absence of exogenously added GlcN.

Developmental effects of GlcN. The addition of GlcN to wild-type developing cells inhibited fruiting body formation (Fig. 2). At concentrations of GlcN greater than 20 mM, the severity of the inhibition of development increased. The two GlcN-resistant mutants, MD1021 and MD1022, had different developmental responses to GlcN. MD1021 was unable to aggregate and form fruiting bodies, but its development was rescued by exogenous GlcN (Fig. 2). In this respect, MD1021 behaved like the group C mutant DK741 (23). As in GlcN-induced lysis of vegetative cells, inhibition of fruiting body formation was a specific event mediated only by amino sugars. GlcN and ManN were the only carbohydrates tested which inhibited fruiting body formation, induced lysis, and rescued the mutants MD1021 and DK741 (32). MD1022 was developmentally proficient in the presence and absence of exogenously added GlcN.

Since GlcN induced lysis in vegetative cells and rescued lysis and development in the group C mutants DK731 and MD1021, we considered the possibility that GlcN was inhibiting development in wild-type cells by triggering lysis prematurely and/or augmenting the lysis event, causing enough cells to lyse that the remaining population was unable to carry on development. It has been shown that cells can be

FIG. 3. GlcN induces premature lysis in starving shake-flask cultures. Vegetative cells were harvested, washed once, and resuspended to a density of 2×10^8 cells per ml in MOPS buffer. The cultures were incubated with shaking at 32°C for 48 h. At the indicated times, samples were removed and counted in a Petroff-Hausser counting chamber.

induced to undergo developmental autolysis and myxospore formation by starving them in liquid shake cultures (38). Accordingly, we looked at the effect of GlcN on cells under these conditions. In contrast to vegetatively growing cells, which completely lysed within 3 to 4 h after the addition of GlcN, no significant lysis occurred in starving cells before 12 h (Fig. 3). However, under these conditions of development, GlcN did in fact stimulate a premature and more massive cell lysis event than was seen in normal starving shake flask cultures. In such cultures, cell numbers began to decrease after 24 h. By 48 h, the culture had decreased in cell number from 2×10^8 cells per ml to 1.35×10^7 cells per ml (93.8% lysis). In cultures treated with 50 mM GlcN, 97.5% of the culture had lysed by 18 h, a full 30 h before untreated cultures.

We next examined the relationship between GlcN induction of autolysis and autocide production, since these compounds have already been implicated in the lytic process (46).

GlcN stimulates production of AM1 in vegetative cells. *M. xanthus* DK1622, MD1004, and MD1005 were examined for production of autocide AM1 after the addition of GlcN to the growth medium. Figure 4 shows that untreated wild-type vegetative cells produced 1.5 U of AM1 per 10^9 cells. The addition of GlcN to vegetative cells induced a dose-dependent production of AM1. Up to 4.9 U of AM1 was produced in response to 50 mM GlcN. MD1021 and MD1022 were both resistant to GlcN-induced lysis. The GlcN-resistant mutants did not produce increased amounts of AM1 in response to GlcN (Fig. 4). Thus, there is a strong correlation between the addition of GlcN, cell lysis, and increased production of autocides in wild-type vegetative cells. This correlation is further strengthened by the fact that the two mutant strains resistant to GlcN-induced lysis also do not increase production of autocides in the presence of GlcN.

Effects of GlcN on AM1 production during development. In order to explore the possibility that autocides were being

FIG. 4. GlcN stimulates autocide production in vegetative cells. Vegetative cells were grown in the presence of various amounts of GlcN for 1 h, harvested, and assayed for AM1 lytic activity as described in Materials and Methods. Each value represents the average of three independent determinations. One unit of AM1 is that amount which yields a net diameter of inhibition of 1 mm.

overproduced during development in the presence of GlcN and thus may be responsible for premature lysis and inhibition of fruiting body formation, we examined developing cells for the production of AM1 in the presence and absence of 50 mM GlcN. Figure 5 shows that AM1 levels normally increased to 4.3 U/ 10^9 cells between 18 and 24 h after the initiation of development. This natural increase of AM1 activity correlates with the onset of developmental lysis in the developing population. However, increased levels of autocides appeared 6 to 12 h earlier in the presence of 50 mM GlcN. Figure 5 demonstrates that the strain MD1021 did not show the normal increase in AM1 production that is seen in wild-type cells. The addition of 50 mM GlcN to developing MD1021 cells restored near-normal production of AM1, as well as the ability to form normal spore-filled fruiting bodies. The addition of 50 mM GlcN to MD1022 did not cause an increase in autocide production during development, and autocide levels were comparable to that of the wild-type strain DK1622.

GlcN induces phospholipase activity in vegetative cells. The autocide AM1, a pool of free fatty acids, is believed to be generated by the action of one or more phospholipase enzymes on autocide AMV, which has been identified as phosphatidylethanolamine (19). We investigated the possibility that GlcN was regulating AM1 production by regulating the phospholipase activity acting on AMV or other membrane phospholipids. Since we did not know the exact nature of the phospholipase activity that might be regulated by GlcN, we used an assay that would measure bulk activity as a result of both phospholipase A and lysophospholipase. Figure 6 demonstrates that the addition of 50 mM GlcN to vegetative cells increased phospholipase activity 7.6 times over that of untreated cells. Vegetative phospholipase activ-

FIG. 5. Production of autocide AM1 during development. Vegetative cells were harvested and resuspended to a density of 8×10^9 cells per ml. Twenty 5- μ l drops (2×10^8 cells per drop) were dried into TPM agar plates and allowed to develop. Duplicate plates were harvested at the times indicated and assayed for AM1 activity as described in Materials and Methods. The values represent the averages of three independent determinations. Symbols: ■, DK1622 and 0 mM GlcN; □, DK1622 and 50 mM GlcN; ●, MD1021 and 0 mM GlcN; ○, MD1021 and 50 mM GlcN; ▲, MD1022 and 0 mM GlcN; △, MD1022 and 50 mM GlcN.

ity did not increase in the mutant strains MD1021 and MD1022 in the presence of GlcN but remained at the uninduced level. These results are consistent with the lysis and autocide production data in that the strains that are resistant to GlcN-induced lysis did not show increased phospholipase activity and also did not lyse or show an increase in autocide production in response to added GlcN.

Phospholipase activity during development. Figure 7 shows phospholipase activity as a function of developmental time. In the wild-type strain DK1622, a 15.1-fold increase in activity was observed between 6 and 12 h after the initiation of development. This activity occurred just prior to the onset of lysis and the increase in AM1 activity during development. Phospholipase activity remained at the higher level throughout development. The presence of 50 mM GlcN stimulated phospholipase activity 1.5 times over that of normal wild-type levels at 6 h of development. MD1021 did not demonstrate the normal increase in phospholipase activity at 6 to 12 h of development unless exogenous GlcN was added. The addition of 25 to 50 mM GlcN to developing cells restored normal developmental kinetics and levels of phospholipase activity and also restored the ability to form fruiting bodies and sporulate. MD1022 showed the normal increase in phospholipase activity during development. As in the cases of fruiting body formation, lysis, and autocide production, phospholipase activity also remained unaffected by GlcN during MD1022 development.

GlcN induces sporulation in vegetative cells. In most cases studied, lysis has always been accompanied by sporulation (23, 40, 42, 49). Since GlcN induced lysis in vegetative cells (Fig. 1), we investigated the possibility that GlcN-induced

FIG. 6. GlcN induces phospholipase activity in vegetative cells. Vegetative cells (5×10^8 cells per ml) were harvested and osmotically lysed. The crude lysate was used for phospholipase determinations as described in Materials and Methods. Phospholipase activity is expressed as nanomoles of lipid phosphorus converted to water-extractable form per minute per milligram of protein. The values represent the averages of three independent determinations.

lysis was also accompanied by sporulation. Figure 8A shows that GlcN-induced lysis was indeed accompanied by the appearance of heat- and sonication-resistant myxospores. Approximately 70 to 75% of the vegetative population lysed in the presence of 15 mM GlcN, and 60 to 70% of the surviving cells sporulated. Microscopic examination of the culture at 6 h revealed the presence of spheroplasts which had lysed by 6 h, while the first myxospores were seen at 12 h after the addition of GlcN and sporulation was complete by 24 h. Those cells which did not lyse or convert to myxospores continued to grow vegetatively.

Two strains that are resistant to GlcN-induced lysis, MD1021 and MD1022, were tested for the formation of myxospores in the presence of 50 mM GlcN. In the presence of 50 mM GlcN, a concentration that normally completely lyses an entire culture of cells, both mutant strains showed no evidence of lysis or sporulation (32). Lower concentrations of GlcN gave the same results (32). These results were consistent with the premise that lysis is required for GlcN-induced sporulation.

GlcN-induced sporulation depends on the ratio of yellow- and tan-phase variants present in the population. *M. xanthus* undergoes a phase variation that can be detected by observing a switch in colony color from yellow to tan (7, 8). Although it is not known whether or not this phase variation plays a role in development, it has become evident that tan cells play a role in GlcN-mediated events. Tan cells were found to be required for GlcN-mediated rescue of lysis and sporulation in *csg* and group C sporulation mutants (23). Tan cells were also found to be more resistant to GlcN-mediated lysis (Fig. 1). Table 1 shows that tan cells were also required for GlcN-induced sporulation. In a cell population containing 100% yellow- or 100% tan-phase variants, GlcN-induced

FIG. 7. Phospholipase activity during development. Developing cells were harvested from TPM agar plates at various times during development and osmotically lysed. The crude lysate was used for phospholipase determinations as described in Materials and Methods. Phospholipase activity is expressed as nanomoles of lipid phosphorus converted to water-extractable form per minute per milligram of protein. The values are averages of three independent determinations. Symbols: ■, DK1622 and 0 mM GlcN; □, DK1622 and 50 mM GlcN; ●, MD1021 and 0 mM GlcN; ○, MD1022 and 50 mM GlcN; ▲, MD1021 and 0 mM GlcN; △, MD1022 and 50 mM GlcN.

sporulation was 4.5% or less. However, sporulation efficiency increased to 70% of the surviving cells if the yellow-to-tan ratio was 90% yellow and 10% tan.

Sporulation efficiency depended upon the concentration of GlcN added and the ratio of yellow- and tan-phase variants present in the population. Table 1 shows that lower concentrations of GlcN (7.5 to 10 mM) stimulated maximum sporulation in populations that were predominantly yellow. For example, 10 mM GlcN stimulated 72% sporulation of surviving cells in a population containing 90% yellow variants and less than 0.01% in populations with 10% yellow variants. At higher concentrations of GlcN (15 to 20 mM), there was a slight increase in sporulation in populations that were predominantly tan (Table 1). For example, 20 mM GlcN stimulated only 2% of the survivors to sporulate in a population containing 10% tan cells and stimulated 12.3% sporulation when tan cells comprised 90% of the population. The fact that tan populations sporulate with slightly better efficiency than yellow populations in higher concentrations of GlcN may be a reflection of their increased resistance to GlcN-induced lysis, with more tan cells surviving lysis to sporulate.

GlcN-induced sporulation is dependent on initial cell density. If cell lysis released a product or a signal required for sporulation, one would predict that the efficiency of sporulation would be related to the initial cell density. Table 2 demonstrates that sporulation occurred only in those experiments in which the cell density was above 2×10^6 cells per

FIG. 8. GlcN induces sporulation in vegetative cells. Vegetative cells were harvested, washed once, and resuspended to a density of 2×10^8 cells per ml in CTT medium supplemented with GlcN. The culture was incubated at 32°C with shaking for 30 h. Samples were removed every 6 h for determination of viable cell number and the presence of viable and heat- and sonication-resistant myxospores. (A) Wild-type cells (DK1622) supplemented with 15 mM GlcN; (B) MD1004 cells supplemented with 50 mM GlcN. Symbols: ●, cells; ■, spores.

ml and 50 to 75% of the cells had been lysed. Low-density cultures (2×10^4 to 2×10^5 cells per ml) did not sporulate even though enough GlcN (1 mM) was added to stimulate 68% lysis (32). It may be that the amount of lysis in the low-density cultures did not release a sufficient concentra-

TABLE 1. Dependency of GlcN-induced sporulation on the ratio of yellow- to tan-colony-phase variants

Yellow/tan ratio ^a	No. of spores (%) at GlcN concn (mM) of ^b :					
	0	7.5	10	15	20	30
100:0	—	4.3 × 10 ⁵ (0.86)	1.1 × 10 ⁶ (2.2)	—	—	—
0:100	—	—	—	2.1 × 10 ⁶ (4.5)	—	—
90:10	—	2 × 10 ⁶ (4.1)	3.4 × 10 ⁷ (72.0)	9.6 × 10 ⁶ (19.7)	9.9 × 10 ⁵ (2.0)	—
10:90	—	—	—	1.9 × 10 ⁷ (39.0)	6.4 × 10 ⁶ (12.3)	—

^a Liquid cultures of yellow- or tan-colony variants were started from individual yellow or tan colonies grown on CTT plates. Ratios of yellow to tan variants were premixed from separate liquid cultures to a density of 2×10^8 cells per ml in CTT medium supplemented with GlcN.

^b Results given in number of viable spores per milliliter present 24 h after the addition of GlcN. Values in parentheses are percentages of the surviving population that sporulated. Dashes indicate less than 0.01% sporulation.

tion of lysis products to stimulate the survivors to sporulate. Apparently, the overall density of the culture, as well as the amount of lysis, plays a role in GlcN-induced sporulation.

The effects of GlcN on sporulation during development. The addition of 30 to 50 mM GlcN to developing cells has been shown to inhibit fruiting body formation and sporulation. These concentrations of GlcN stimulate a massive and premature lytic event in developing cells, leaving the surviving cells at too low a cell density to form fruiting bodies or sporulate. Lower concentrations of GlcN had different effects when tested on wild-type developing cells. Table 3 shows that sporulation was enhanced 11-fold in the presence of 10 mM GlcN, while 20 mM GlcN had an inhibitory effect on sporulation. The presence of both enhancement and inhibition of sporulation responses to GlcN demonstrates that a delicate balance between lysis and sporulation exists. The enhancement of sporulation could be due to the fact that GlcN also causes the release of the myxobacterial autocide AM1. The addition of AM1 has been shown to enhance sporulation (37).

The mutant strains MD1021 and MD1022 had different developmental responses to GlcN even though growing cells of both strains were resistant to GlcN-induced lysis. Development in MD1021, which was deficient in fruiting body formation and sporulation, was rescued by GlcN; i.e., the addition of 20 to 50 mM GlcN restored sporulation to wild-type levels. GlcN also rescued deficiencies in autocide production, phospholipase activity, and fruiting body formation. MD1022 was vegetatively and developmentally resistant to the effects of GlcN. Sporulation was not enhanced or inhibited by the addition of 50 mM GlcN to developing MD1022 cells.

GlcN stimulates glycerol production in vegetative cells. During thin-layer chromatography analysis of ethanolic extracts of GlcN-treated cells, a spot with the R_f of glycerol was observed (32). This spot was not present in extracts of untreated cells. Since glycerol is known to induce sporula-

tion in vegetative cells, we investigated more fully the relationship between GlcN induction and the subsequent intracellular accumulation of glycerol. The time course of accumulation of glycerol in growing, vegetative cells treated with 50 mM GlcN was determined by HPLC. Figure 9 shows that after 30 min of incubation with GlcN, intracellular levels of glycerol rose from a basal level of 90 nmol/ 10^8 cells to 170 nmol/ 10^8 cells. The increased level of glycerol was transient. After a 2-h incubation with GlcN, glycerol levels fell below the initial basal level of 90 nmol/ 10^8 cells. Analysis of the time course of the appearance of glycerol in response to GlcN was repeated by using gas-liquid chromatography analysis, and the results were the same as described above (32).

Comparison of myxospores derived from fruiting bodies and those induced by glycerol and GlcN. We used several of the properties of myxospores to compare GlcN-induced spores with those induced by glycerol and those derived from fruiting bodies. Spores isolated from fruiting bodies as well as glycerol-induced spores are optically refractile (17) and heat and sonication resistant (44). GlcN-induced spores are also optically refractile and heat and sonication resistant (32). Although fruiting body-derived spores and glycerol spores share similar properties, they differ markedly in biochemical and ultrastructural characteristics (22). In order to compare ultrastructural characteristics, spores were isolated, sectioned, stained, and observed by transmission electron microscopy. Figure 10A shows a section of a spore derived from a mature (96-h) fruiting body. It has a thick spore coat consisting of a number of distinct layers, which is characteristic of spores derived from fruiting bodies (22). Figure 10B shows a glycerol-induced spore. It is markedly different from fruiting body spores in that, characteristically, it has only a thin, single-layered spore coat. A GlcN-induced spore is shown in Fig. 10C. It clearly resembles the glycerol-induced spore in that it, too, has one thin spore coat.

TABLE 2. Effects of GlcN on sporulation during development^a

Strain	No. of spores ^b at GlcN concn (mM) of:				
	0	10	20	30	50
DK1622	6.8×10^6	7.5×10^7	3.2×10^5	—	—
MD1004	—	—	8.3×10^6	1.1×10^7	9.6×10^6
MD1005	5.2×10^6	6.3×10^6	6.2×10^6	4.0×10^6	4.5×10^6

^a Vegetative cells (90% yellow and 10% tan) were harvested and resuspended to a density of 8×10^9 cells per ml. Cells were spotted onto TPM agar plates (25 μ l per drop, 20 drops per plate) and allowed to develop. Duplicate plates were harvested after development for 96 h and assayed for heat- and sonication-resistant myxospores.

^b Number of viable spores per 25- μ l drop. Dashes indicate less than 0.01% sporulation.

TABLE 3. Dependency of GlcN-induced sporulation on the initial cell density

No. of initial cells/ml ^a	No. of surviving cells/ml ^b	% Lysis	No. of spores/ml ^c	% Sporulation ^d
2×10^4	0	100	0	0
2×10^5	0	100	0	0
2×10^6	1.6×10^5	92.0	8.8×10^4	55.0
2×10^7	5.3×10^6	73.5	3.3×10^6	62.0
2×10^8	9.6×10^7	52.0	1.6×10^7	16.6
2×10^9	1.9×10^9	0.5%	0	0

^a Vegetative cells (90% yellow and 10% tan) were harvested, washed, and resuspended to different densities in CTT growth medium containing 10 mM GlcN.

^b Surviving cells were measured 6 h after the addition of 10 mM GlcN.

^c Viable spores were measured 24 h after the addition of 10 mM GlcN.

^d Represents the percentage of survivors that sporulated.

DISCUSSION

Having defined the effects of the amino sugar GlcN on lysis and sporulation in *M. xanthus*, we propose that GlcN stimulates the activity of one or more phospholipases which in turn hydrolyze membrane phospholipids. The products of this hydrolysis, free fatty acids (AM1) and glycerol, may be involved in mediating the developmental processes of lysis and sporulation, respectively. On the basis of a series of close correlations, we propose that GlcN-induced lysis may involve mechanisms similar to those involved in developmental autolysis. It may, therefore, serve as a model system for examining developmental autolysis more closely.

The addition of GlcN to vegetative and developing cells

induced a rapid, synchronous, and massive lytic event. This effect was specific for the amino sugars GlcN and ManN (both of which have been shown to rescue developmental autolysis in the group C developmental mutant [23]) and is not induced by any other sugars we have tested. GlcN-mediated lysis occurs in vegetative cells in the presence of chloramphenicol and rifampin (32), suggesting that no new protein synthesis is required to effect GlcN-mediated lysis.

GlcN induced not only lysis but also sporulation in growing vegetative cells. The finding that GlcN also resulted in a transient increase in the intracellular concentration of glycerol has also allowed us to rationalize GlcN-induced sporulation on the basis of the fact that glycerol is a known sporulation inducer (17).

GlcN may mediate lysis via the induction of myxobacterial autocides such as AM1, since the addition of GlcN induced the release of free fatty acids that were chromatographically identical to the free fatty acids of AM1 (32). We have demonstrated that AM1 levels increased naturally during development between 18 and 24 h after the initiation of starvation. This increase in autocide production occurred concomitantly with the onset of developmental lysis. The addition of GlcN accelerated the formation of autocides, and they appeared earlier than normal, 6 to 8 h after the initiation of starvation. The increased amounts of autocides, as well as their premature appearance, may lyse enough of the original population to render the survivors incapable of continuing with development and fruiting body formation.

Gelman et al. suggested that AM1 free fatty acids are released as a result of the hydrolysis of another autocide, AMV, by one or more phospholipases (19). Phospholipase activity appeared during normal development 6 to 12 h before the appearance of AM1 and the onset of developmental lysis. The addition of GlcN caused an increased stimulation of phospholipase activity 6 h after the onset of development. This is consistent with the fact that starving cells did not show signs of lysis until at least 6 h after the addition of 50 mM GlcN. The activity of several lipases and phospholipases in *M. xanthus* have been observed. Glycerol ester hydrolase activity has been observed (43), but it may only be involved in lysing prey bacteria, and a developmental role has yet to be demonstrated. Orndorff and Dworkin demonstrated membrane-bound phospholipase A and lysophospholipase activity (34), while Gelman et al. found phospholipase activity in the culture supernatant (19). The developmental role of phospholipase activity in *M. xanthus*, as suggested by us, is in contrast to the functions of phospholipase enzymes in other bacterial systems. Studies on membrane-bound phospholipases in *Escherichia coli* have shown no evidence

FIG. 9. GlcN stimulates glycerol production in vegetative cells. Vegetative cells were harvested, washed once, and resuspended to a density of 2×10^8 cells per ml in CTT growth medium containing 50 mM GlcN. Ten milliliters of culture was removed at the indicated time points, and the sample was assayed for glycerol content as described in Materials and Methods. The values are given in nanomoles of glycerol per 1.2×10^8 cells and represent intracellular glycerol levels only.

FIG. 10. Electron micrographs of thin sections of myxospores. (A) Fruiting body spore; (B) glycerol-induced spore; (C) GlcN-induced spore.

for a physiological role for these enzymes (12). Mutants defective in the structural genes for outer membrane phospholipase A (15) show no effect of these mutations on growth. The best-characterized bacterial phospholipase enzymes are the extracellular phospholipases found in *Clostridium*, *Bacillus*, and *Staphylococcus* species; these seem to function mainly as virulence factors (1).

The effects of GlcN on the GlcN-resistant mutants have shed some light on how GlcN regulates the lysis response. MD1021 and MD1022 were isolated on the basis of their abilities to grow vegetatively in the presence of lethal amounts of GlcN, and yet they have different developmental responses to GlcN. MD1021 had the unexpected trait of being vegetatively resistant to GlcN-induced lysis and yet requiring GlcN to complete development. It is not clear whether this reflects different responses to GlcN or to lysis. During development, GlcN rescues fruiting body formation, phospholipase activity, autocide production, and sporulation in the MD1021 mutant. This is consistent with our model in which GlcN regulates a lysis and sporulation pathway that includes phospholipase activity and autocide and glycerol release. The inability of GlcN to induce MD1021 vegetative cells to lyse may suggest an alteration (possibly in the cell surface) that renders the growing vegetative cell less accessible or sensitive to GlcN. MD1022, both vegetative and developing cells, is refractile to the effects of GlcN. This, too, may reflect a change in the cell surface that renders the cells incapable of responding to GlcN.

GlcN has not been shown to be active in cell-cell signaling in other biological systems. In fact, nonacetylated GlcN is rare and unusual in other glycoconjugates. It has been described as a component of a plant phytylglycolipid (10) and has been found in several polymeric structures, such as the chitosan of several fungi (2, 13). GlcN is also found in the cell wall peptidoglycan of some bacteria (31). More recently, GlcN has been found to be a major component in the glycosyl phosphatidylinositol membrane anchors that form the basis of attachment of many proteins to cell surfaces in some eukaryotic organisms (14). It is not known in what form, if any, GlcN is naturally present during development. It may be present as part of a larger signal molecule such as DS, a cell-associated factor containing carbohydrate components that also regulates lysis during development (23).

The role of lysis in myxobacterial development has been a

persistent question. While other studies have shown that lysis and sporulation are connected with developmental events (23, 48, 49), this view has been challenged (33). There are two questions: (i) does autolysis occur as a normal part of the developmental process, and, if so, (ii) what is its function? The work of Rosenbluh et al. (36) has provided strong evidence that lysis does indeed occur as a normal event during development. With regard to the function of developmental lysis, it has been proposed that lysis is required for sporulation. We have shown that GlcN-induced lysis and sporulation are linked and interdependent processes. Using the strains MD1021 and MD1022, which are resistant to GlcN-induced lysis, we have shown that GlcN-induced sporulation did not occur in the absence of lysis. This is consistent with the observation that all strains that are deficient in lysis are also unable to sporulate (40). The fact that the addition of GlcN alone stimulated both lysis and sporulation also argues that lysis and sporulation are connected events. While it has been suggested that the function of lysis is to release nutrients, developmental signals, or substrates which are required for sporulation (45, 48, 49), Rosenbluh and Rosenberg (39) have recently proposed that the autocide AM1 functions to increase the permeability and receptivity of developing cells to intercellular signals. They suggest that lysis is a subsequent result of this process.

In the past, it has not been clear whether or not there was a relationship between glycerol as an exogenous inducer of sporulation and the natural inducer of sporulation. Because the other events that occur in response to added GlcN (lysis, sporulation, autocide production, and phospholipase activity) were found to occur normally, we propose that glycerol, which is also made in response to GlcN, may be a naturally active compound that plays a role in the sporulation process. Studies are under way to see whether glycerol is naturally produced during development. A rough calculation indicated that the glycerol concentration in vegetative cells was very high (0.25 M). After treatment with GlcN, the glycerol levels rose to approximately 0.5 M, the concentration needed to induce vegetative cells to sporulate by adding exogenous glycerol (17). This shows that, theoretically, enough glycerol was present intracellularly to induce sporulation.

It has been shown that glycerol-induced spores lack the multilayered coat characteristic of spores isolated from fruiting bodies, presumably because glycerol bypasses the

FIG. 11. Proposed model for GlcN regulation of lysis and sporulation.

normal requirements for development (22). Like glycerol induction, GlcN induction also bypasses the requirements of starvation, a solid surface, and multicellular development and initiates lysis and sporulation directly. Thus, it is not surprising that the spores produced in response to glycerol and GlcN resembled each other ultrastructurally. It is interesting that while both glycerol and GlcN stimulate sporulation, they seem to initiate the pathway at different points. Glycerol induction is independent of cell density and does not induce any lysis in the population. While GlcN also bypasses the requirement for nutritional downshift, it initiates the sporulation process at the point of lysis (which is density dependent [36]) and thus still requires the presence of a minimum cell density which can support both the lysis and sporulation process.

During morphogenesis in *M. xanthus*, two cell fates have been observed to occur within the maturing fruiting body. Either the cells lysed or they survived and sporulated. What determines which cells undergo which fate? One plausible strategy for the cells is to regulate the amount of lysis signal released so that only a certain proportion of the cells are lysed and the survivors sporulate. In the GlcN system, the amount of GlcN (lysis signal) added does in fact have a direct effect on the amount of subsequent sporulation. Another way of ensuring that enough cells always survive lysis and sporulate is for there to be a subpopulation of cells resistant to the lysis signal itself. There is now some evidence that the tan-phase variants in the myxobacterial population may serve this function. Janssen and Dworkin first demonstrated that tan cells were required for the rescue of the group C class mutants by either the cell-associated factor DS or the amino sugars GlcN or ManN (23). It has also been proposed that tan cells preferentially form myxospores (40). The results presented in this paper show that tan cells did indeed respond differently to GlcN than did yellow cells. First, tan cells were found to be more resistant to GlcN-induced lysis than were yellow cells (32). Second, tan cells were found to be required for GlcN-induced sporulation. Within the framework of our model, we suggest that GlcN preferentially lyses yellow cells, while the tan cells, being more resistant to GlcN-mediated lysis, survive and sporulate, possibly responding to liberated glycerol.

In Fig. 11, we have proposed a model, based on work presented in this paper, that describes the sequence of events that begins with GlcN-induced lysis and culminates with sporulation. We propose that the addition of GlcN eventually results in the generation of two types of signals,

one which regulates the sporulation response and another which regulates lysis. The two most likely candidates for these signals are glycerol and the autocide AM1 (free fatty acids), both of which can be induced by the addition of GlcN and can be produced via the hydrolysis of a single compound, a membrane phospholipid. We have shown that GlcN will stimulate increased phospholipase activity in *M. xanthus*. Therefore, we suggest that GlcN initiates lysis and sporulation by acting as an initial signal which stimulates one or more membrane phospholipases to hydrolyze membrane phospholipids. The hydrolysis products, AM1 free fatty acids and glycerol, in turn act as second messengers and mediate the developmental events of lysis and sporulation, respectively. It has not escaped our attention that this model is reminiscent of the pathway of eukaryotic signal transduction by which an environmental or hormonal signal triggers the hydrolysis of the membrane phospholipid phosphatidylinositol. The hydrolysis products, inositol triphosphates and diacylglycerol, in turn perform second messenger functions by mobilizing calcium and regulating protein kinase activity (3).

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