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Function of Ammonium Transporter A in the Initiation of Culmination of Development in *Dictyostelium discoideum*

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The histidine kinase DhkC controls a phosphorelay involved in regulating the slug versus culmination choice during the multicellular developmental program of *Dictyostelium discoideum*. When the relay is active, slug migration is favored due to the activation of a cyclic AMP (cAMP) phosphodiesterase and the resultant lowering of the intracellular and extracellular levels of cAMP. Ammonia signaling represents one input into the DhkC phosphorelay, and previous studies indicated that the ammonium transporter C inhibits the relay in response to low ammonia levels. Evidence is presented that another member of the family of ammonium transporters, AmtA, also regulates the slug/culmination choice. Under standard conditions of development, the wild-type strain requires a transitional period of 2 to 3 h to go from fingers to culminants, with some slugs forming and migrating briefly prior to culmination. In contrast, *amtA* null cells, like cells that lack DhkC, possessed a transitional period of only 1 to 2 h and rarely formed slugs. Disruption of *amtA* in an *amtC* null strain overcame the slugger phenotype of that strain and restored its ability to culminate. Strains lacking AmtA were insensitive to the ability of ammonia to promote and prolong slug migration. These findings lead to the proposal that AmtA functions in ammonia sensing as an activator of the DhkC phosphorelay in response to perceived high ammonia levels.

Amoebae of *Dictyostelium discoideum* foraging on bacteria within the leaf litter of deciduous forests initiate a multicellular developmental program in response to a lack of food source. The goal of the program is to generate and disperse spores so that some cells might survive and escape the unsupportive environment. As the cells aggregate and form the initial multicellular structures, or mounds, differentiation occurs, giving rise to several cell types: prespore cells and prestalk A and O cells (defined by expression of different promoter regions—*ecmA* and *ecmO*—of the *ecmA* gene) (14). Subsequent cell sorting and patterning occurs as tipped mounds form: prestalk A cells localize to and constitute the anterior-most portion of an apical tip; a band of prestalk O cells forms under the prestalk A cells to make up the lower portion of the tip; and the remaining 70 to 80% of the cells posterior to the tip are mostly prespore cells. The apical tip of prestalk cells is referred to as an organizer because it controls patterning and subsequent morphological events (6, 9, 20, 33).

The tipped mound elongates into the cylindrical first finger stage in which the spatial arrangement of cell types is retained. The first finger may initiate culmination, in which morphological changes and differentiation of precell types to mature stalk cells and spores occurs to produce the fruiting body, with a spore-filled sorus sitting on top of a cellulosidic stalk. Alternatively, the finger may transition into a slug that migrates until environmental conditions conducive for spore dispersal are sensed. The ability to sense a favorable environment, and hence determine when culmination is appropriate, is mediated by cells within the anterior-most tip of the finger and slug (30). The term transitional period is defined as the time when fingers are transitioning to early culminants, including the variable time spent, if any, as a migrating slug.

At the end of the transitional period, the initiation of culmination is manifest by the formation of a small cone of prestalk AB cells (marked by expression of *ecmB*) embedded within the prestalk A region (14, 15). The prestalk AB cells derive from a subset of the prestalk A cells at the most anterior tip, termed prestalk A* cells (12). Prestalk AB cells produce a nascent stalk tube into which surrounding prestalk A cells are recruited while the tube elongates toward the substratum (25). The maturing stalk cells within the tube secrete factors that signal to the prespore cells to begin differentiation into mature spores (1, 2), and the maturing spore mass begins moving up the stalk.

The transitional period is influenced by a number of environmental factors, such as humidity, light, temperature, and others (3, 18, 21, 29), that presumably reflect appropriate or inappropriate surroundings for maximizing spore dispersal (23, 31). One means for monitoring the local environment for its suitability for spore dispersal is through the volatile compound ammonia that is produced and subsequently sensed by the developing cells (23). Low local ammonia levels promote culmination, while high concentrations result in slug migration due to their indication of a poor environment for spore dispersal (31). Ammonia is thought to mediate the alternative outcomes of slug migration versus culmination in part via modulation of cyclic AMP (cAMP)-dependent protein kinase A (PKA) (13, 24, 28). Previous work demonstrated that the histidine kinase DhkC controls PKA activity to regulate the slug/culmination choice (16, 28). This is accomplished via a phosphorelay that modulates the activity of the cAMP phosphodiesterase RegA. High ammonia levels result in an ac-

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tive phosphorelay, the activation of RegA, and the inhibition of PKA activity and culmination.

Kirsten and coworkers demonstrated a role for the ammonium transporter C protein (AmtC) in controlling the DhkC phosphorelay (16). Strains lacking AmtC do not culminate, and the resultant slugger phenotype is due to an inability to inhibit the DhkC phosphorelay. It was postulated that AmtC functions as an ammonia sensor and inhibits the phosphorelay in response to low ammonia levels, thus allowing cAMP accumulation to activate PKA and subsequently initiate culmination (16).

Within the tips of *amtC* null slugs, expression of *ecmB*, the marker for the initiation of culmination, is blocked. Additionally, STATa-dependent CudA expression in the tip is blocked because STATa nuclear localization does not occur (16). CudA (*cu*lmination *d*eficient) is a nuclear protein whose expression in prestalk A* and prestalk AB cells is induced by the transcription factor STATa (12), and whose presence in these cells is required for culmination (11). CudA also is expressed in prespore cells by a STATa-independent mechanism. Nuclear localization of STATa in tip cells normally results from tip-specific production of extracellular cAMP by adenylyl cyclase A (5, 32). Although adenylyl cyclase A expression is misregulated in the *amtC* null strain, it is expressed in tip cells, yet STATa is not localized to the nuclei. Thus, while cAMP is being produced in tip cells during the transitional period of the *amtC* mutant strain, an overly active DhkC phosphorelay apparently results in the rapid degradation of cAMP, such that intracellular and extracellular levels do not build up to induce nuclear localization of STATa nor to activate PKA, both of which are required to initiate culmination. This was confirmed by the restoration of STATa nuclear localization, CudA expression, and rescue of the slugger phenotype of the *amtC* null strain when the DhkC phosphorelay was inactivated by additionally knocking out either *dhkC* or *regA* (16).

Herein we report findings on mutant strains lacking another member of the family of putative ammonium transporters, AmtA. Evidence is presented that supports the postulate that AmtA is also an ammonia sensor that regulates the DhkC phosphorelay and the slug/culmination choice. AmtA is proposed to promote slug formation by activating the phosphorelay in response to high ammonia levels.

MATERIALS AND METHODS

Cell growth and development. *Dictyostelium discoideum* strain Ax4 was used as the wild-type strain in all experiments. Cells were grown axenically in a rich broth (HL-5 media) or on SM plates with *Klebsiella pneumoniae* (27). Standard development was performed as described previously (26, 28) using cells that had been grown in the presence of bacteria as a food source, which was removed by low-speed centrifugation. Development in the presence of exogenously added ammonia was carried out as described previously (28) by transferring early fingers to pads soaked with 100 mM ammonium hydroxide titrated to pH 7.5 with concentrated phosphoric acid.

Disruption of the *amtA* gene. To disrupt the *amtA* gene (dictyBaseID, DDB0185017), a 2,244-bp region of the coding region and upstream sequence was amplified from genomic DNA using the following primers: GGTTCATCA TCAGTTGTTC and TATGTATGTTGAAACCTCTGC. The resultant fragment was cloned into the pGEM-T vector (Promega) and digested with MfeI to remove a 375-bp region near the middle of the *amtA* fragment, resulting in the deletion of the coding region for amino acids 237 through 362 (40% of the transmembrane spanning domains). Either a blasticidin resistance cassette (8) or a hygromycin resistance cassette (7) was inserted to replace the deleted coding region. The resulting plasmids were digested with EcoRI to release the *amtA* disruption cassette, and the digests were transformed into exponentially growing Ax4 cells (17, 28). Transformants were selected at 20 μg/ml for blasticidin or 30 μg/ml for hygromycin. Clonal isolates were obtained, and disruption of the *amtA* gene was confirmed by PCR using one primer in the disrupted gene external to the integration site and one from the selection cassette. Multiple independent isolates were obtained, and they all possessed the same phenotype when developed under standard conditions. One of the strains, BS155 (blasticidin resistant), was used in the experiments shown. Disruption of the *amtA* gene in the *amtC* null strain BS154 was made using the hygromycin disruption cassette for *amtA*, as the *amtC* gene is disrupted in this strain with a blasticidin cassette. Several independent clones were obtained and demonstrated to contain the disrupted *amtA* gene. Three independently derived strains were examined for development, and all showed the same phenotype; that is, fruits were formed. One of these double null strains (BS165) was used for most of the experiments presented herein. For all *amtA* null strains (single and double nulls), no *amtA* mRNA was detectable by reverse transcription (RT)-PCR.

RT-PCR. RNA was isolated from growing cells and from cells at various times after the initiation of development using Trizol (Sigma). RT-PCR was carried out as described previously (19). In all RT-PCRs, oligonucleotides specific for the H7 gene were used as an internal control, as H7 mRNA is expressed at constant levels during growth and all stages of development (34).

Histochemical staining. The *lacZ* construct for the prestalk-specific promoter *ecmAO* was generously provided by K. Jermyn and J. Williams and was transformed into Ax4 and null cells by calcium phosphate precipitation or electroporation. Bluo-gal (Sigma) staining of filter-developed cells was performed as described previously (4) with modifications (22).

Monoclonal anti-STATa (D4) and purified anti-CudA (mAB11) serum were kindly provided by J. Williams and M. Fukuzawa. Slugs that had migrated from 1 to 3 h were used for immunohistochemical staining after being developed and harvested as described previously (16).

Microscopy and image processing. Standard development and β-galactosidase staining results were photographed with a Leica MZ16 stereomicroscope with a Q-Imaging Retiga 1300 camera and Q-Imaging or Simple PCI software. Fluorescent immunohistochemical results were photographed with an Olympus AX70 compound microscope with a Q-Imaging Retiga EXi camera and OpenLab software or on a Zeiss LSM510 inverted confocal microscope with Zeiss laser scanning microscope software. Confocal projections of Z-stacks and reslicing to produce new stacks were done with ImageJ (NIH). Figures were prepared with PhotoStudio (ArcSoft).

RESULTS

Disruption of *amtA* results in subtle abnormalities. While disruption of the *amtC* gene results in severe developmental aberrations (10, 16), disruption of the *amtA* gene resulted in only minor defects when the cells were starved and placed under standard conditions of development (Fig. 1). Initial developmental timing was similar to that of the wild type, but aggregates and fingers were smaller than normal. Little time was spent in the transitional period, with slug formation (fingers falling to the substratum) being rare, and those slugs that did form did not migrate but immediately rose to become second fingers. *amtA* null fingers began culmination within 1 to 2 h after their formation. Although standard conditions of development provide an environment that minimizes slug formation/migration, some "indecision" is normally seen under these conditions, as revealed by a transitional period of 2 to 3 h in the wild type Ax4 strain, with the majority of fingers falling over and briefly migrating prior to culminating. While slugs were rare for the *amtA* null strain under standard conditions of development, slugs formed and migrated when the cells were developed under conditions that promote slug formation (not shown). Culmination of the null strain occurred with comparable timing to that of Ax4 but was asynchronous, with mixes of early, mid, and late culminants often observed. The final fruiting bodies had normal morphology except for being small but normally proportioned (Fig. 1).

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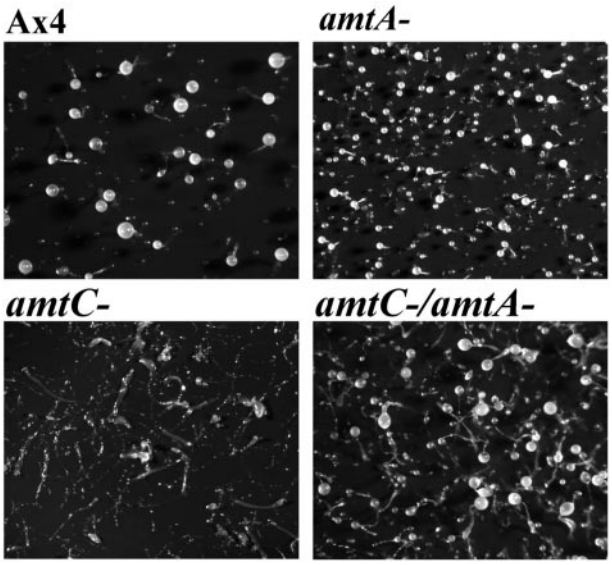

FIG. 1. Developmental morphology of the *amtA*, *amtC*, and *amtC/amtA* null strains and the parental strain Ax4. Cells of each strain were plated for development under standard conditions and photographed at 28 h poststarvation.

Interestingly, *amtA* null cells possessed a rapid growth phenotype when grown axenically in shaking cultures (Fig. 2). The average doubling time for *amtA* null cells was 10.4 h, while that for Ax4 cells was 11.2 h. The growth rates of the other ammonium transporter null strains, *amtC* null and *amtB* null, were essentially identical to that of the parental Ax4 cells, as were the growth rates of all three combinations of the double nulls.

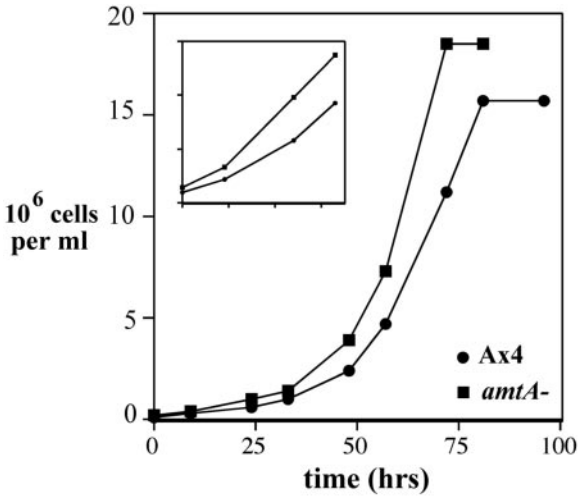

FIG. 2. Growth curves for the *amtA* null strain and the parental strain Ax4. Axenic cultures were started at 2×10^5 cells per ml and shaken at 21°C. Cells were counted at the indicated times. The inset shows the starting titer and the first three time points at a different scale to demonstrate that the rate difference was observed throughout the growth of the culture. Several independent experiments were performed using different starting titers. The average doubling times during exponential growth from four such experiments were 10.4 h for *amtA* null cells and 11.2 h for Ax4 cells. *amtA* cultures averaged a 15 to 20% higher cell titer at the stationary phase.

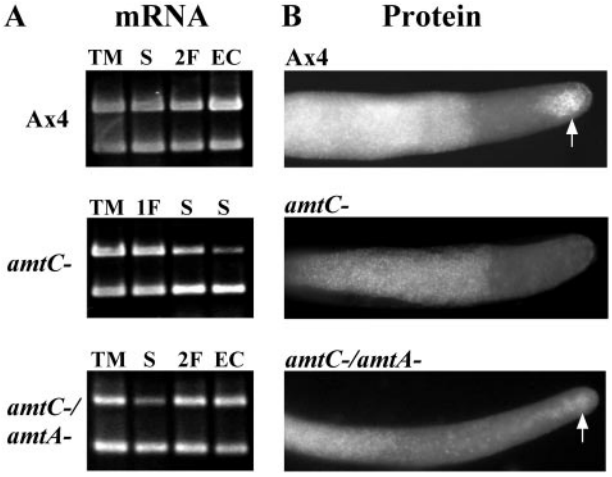

FIG. 3. Expression of CudA in the *amtC/amtA* double null strain and the parental Ax4 and *amtC* null strains. Growing cells of each strain were harvested and plated for development under standard conditions. Structures were collected after various times of development, and either RNA was isolated for RT-PCR analysis (A) or the multicellular structures were fixed and processed for immunohistochemical staining for the CudA protein (B). RT-PCR was performed using primers for the *cudA* gene (top band in each panel) and the constitutively expressed *H7* gene (bottom band) as a control. TM, tipped mound; 1F, first finger; S, slug (just formed and 6 h later for the *amtC* null strain); 2F, second finger; EC, early culminant. For spatial localization of CudA during the transitional period, slugs were fixed and immunohistochemically stained after 1 to 3 h of migration. Visualization was with the fluorescent secondary antibody Alexa Fluor 568 (Molecular Probes). White regions are the areas of CudA expression; arrows indicate CudA presence in the tip region. Punctate staining is because CudA is a nuclear protein.

Loss of AmtA rescues the *amtC* null slugger phenotype. Disruption of the *amtA* gene within the *amtC* null strain rescued the slugger phenotype of the latter strain (Fig. 1). The rescue was specific for loss of the *amtA* gene, as the *amtC/amtB* double null strain retains the slugger phenotype (unpublished). As with the *amtC* null strain but in sharp contrast to the *amtA* null strain, the *amtC/amtA* double null fingers initially all fell to the substratum to give a field composed only of slugs. However, over the next 2 to 4 h, the double null slugs exhibited little or no migration and, in an asynchronous manner, lifted off the substratum and initiated culmination. In contrast, *amtC* null slugs remain slugs and migrate indefinitely (10, 16). Subsequent culmination of the double null entities occurred with normal timing, so by 26 to 28 h, only fruiting bodies were observed (Fig. 1).

Tip expression of CudA and nuclear localization of STATa are recovered in the *amtC/amtA* double null strain. The inability to inhibit the DhkC phosphorelay in developing *amtC* null cells leads to the lack of localization of STATa within the nuclei of the anterior tip cells and a consequent lack of production of CudA within the pstA*/AB cells (16). Hence, we examined these molecular events in developing *amtC/amtA* null cells. When analyzed by RT-PCR, the double null strain exhibited a reduction in *cudA* mRNA expression during the slug stage that was recovered during culmination (Fig. 3A). This was confirmed with immunohistochemical staining for the CudA protein (Fig. 3B), which importantly showed CudA

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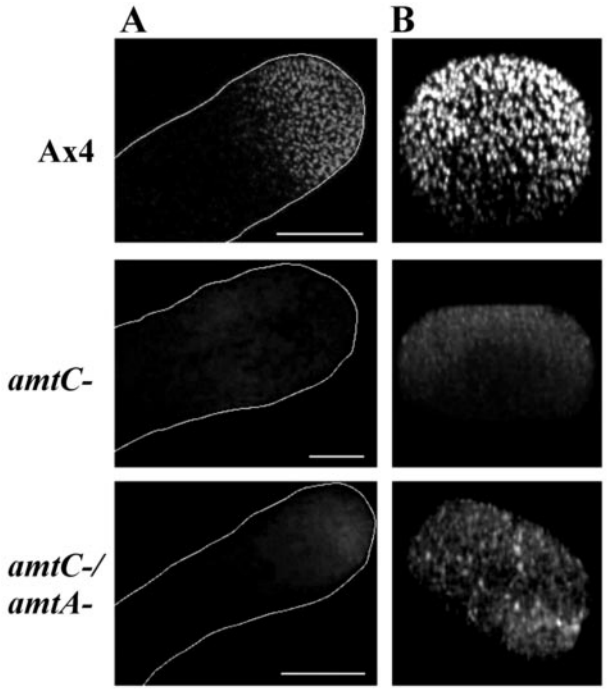

FIG. 4. Confocal analysis of STATa expression in the *amtC*/*amtA* null strain and the parental Ax4 and *amtC* null strains. Growing cells of each strain were harvested and plated for development under standard conditions. Slugs were harvested and fixed after 1 to 3 h of migration, and immunohistochemical staining was carried out for the STATa protein. Visualization was with the fluorescent secondary antibody Alexa Fluor 568 (Molecular Probes). White regions are the areas of STATa expression. Punctate staining indicates that STATa has translocated to the nucleus. Nonpunctate staining indicates that STATa is present within the cytosol. (A) Projections of the original Z-stacks. Bars, 50 μm. (B) ImageJ (NIH) was used to produce a stack of 1-μm slices of the first 25 μm of the prestalk region on a 90° transverse cross-section to generate a new projection of the tip.

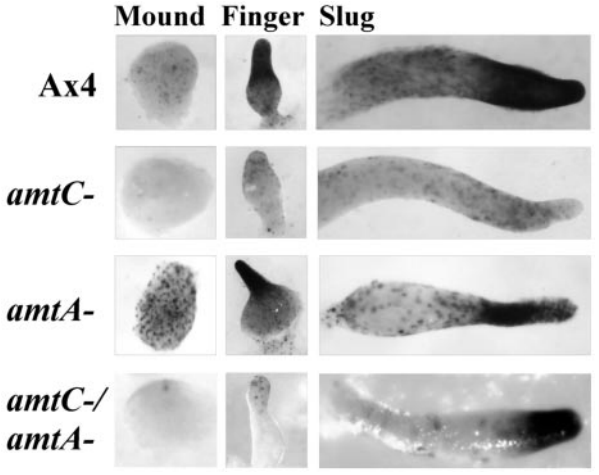

FIG. 5. Expression of the *ecmAO* promoter in the *amtA*, *amtC*, and *amtC*/*amtA* null strains and in the Ax4 parental strain. Cells were transformed with the *ecmAO*::*lacZ* plasmid and plated for development. The developing structures were fixed and stained for β-galactosidase activity. Developmental stages and strains are as labeled. Areas of black indicate expression of the *ecmAO* promoter.

present in the anterior tip cells. Although there was usually a significant loss of CudA expression during slug migration, nuclear localization of STATa was present (Fig. 4) but at considerably reduced levels relative to that of the wild type. In contrast, slugs of the *amtC* null strain exhibited no nuclear localization of STATa (Fig. 4) at any time and thus no STATa-induced CudA expression within their tips (16). The recovery of *cudA* expression to wild-type levels when the *amtC*/*amtA* double null strain began culmination was in contrast to the *amtC* null strain, which continued to have declining levels (Fig. 3A). The presence of CudA in prespore cells, which results from a STATa-independent mechanism, was seen in all three strains but, curiously, also declines during slug migration in both the *amtC* null and *amtC*/*amtA* null strains. Similar to the STATa-dependent tip expression of CudA, prespore expression was restored to wild-type levels at the onset of culmination in the *amtC*/*amtA* double null strain.

Loss of AmtA does not restore initial expression of prestalk genes in the *amtC* null strain. In addition to the slugger phenotype, prestalk cell differentiation and gene expression is significantly altered in developing *amtC* null cells (16). Defects include a substantial delay in both *ecmA* and *ecmO* expression and in prestalk A and prestalk O cell type differentiation. A plasmid expressing the *lacZ* gene under the control of the *ecmAO* promoter was transformed into the single and double null strains and into Ax4 cells. The *amtA* null strain gave an essentially normal pattern of expression, with possible overexpression of *ecmO* relative to *ecmA*, as seen in the early slug stage (Fig. 5).

Similar to the *amtC* null strain, the *amtC*/*amtA* double null strain gave delayed expression of the *ecmAO* promoter, with no detectable expression in mounds and very weak expression from tipped mounds through initial slugs (Fig. 5). However, as slugs began transitioning to culmination, the *ecmAO* promoter reverted to the wild-type pattern of strong expression within the anterior cells, and normal expression was maintained throughout culmination. This is in contrast to the *amtC* null strain in which the *ecmAO* promoter remains greatly underexpressed until several hours after slugs have migrated, and most expression is subsequently lost on further migration (16).

***amtA* null fingers are insensitive to the prolongation of slug migration by ammonia.** The inability to culminate and the lack of proper nuclear translocation of STATa in developing *amtC* null cells are attributable to a misregulated and overly active DhkC phosphorelay (16). AmtC is thought to be an inhibitor of the phosphorelay and has been proposed to be the sensor of low ammonia levels. Given that the loss of AmtA rescues the slugger phenotype and restores nuclear translocation of STATa, we suggest that AmtA also may regulate the DhkC phosphorelay by stimulating the relay and serving as a sensor of high ammonia levels. If true, then cells lacking AmtA would be incapable of perceiving high ammonia levels. The fact that addition of exogenous ammonia to developing cells when they are just forming fingers promotes and prolongs slug migration (23) was used to test this possibility.

Ax4 and *amtA* null cells were developed to the tipped mound/early finger stage under standard conditions, and one filter of each was transferred to pads soaked with ammonia, while one filter remained on pads without ammonia. As seen in

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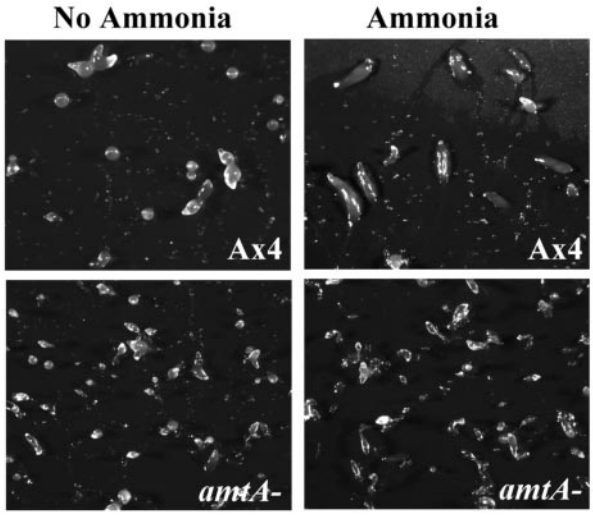

FIG. 6. Exposure of developing *amtA* null cells and the parental Ax4 cells to exogenously added ammonia. Cells were developed under standard conditions until fingers had just begun to form (12 to 13 h poststarvation), at which time half of the filters were transferred to pads soaked in 100 mM ammonium phosphate, pH 7.5. Fingers/slugs generally formed within 1 to 1.5 h, and photographs were taken 6 h after fingers had fully formed, 19 to 21 h poststarvation. Four independent experiments were carried out.

Fig. 6, ammonia prolonged the slug stage for the Ax4 cells, with the time spent in the transitional period increasing from 2 to 3 h to 6 to 7 h. In contrast, the *amtA* null strain had a transitional period of 1 to 2 h independent of the presence or absence of exogenously added ammonia. The *amtC* null/*amtA* null strain also was insensitive to prolongation of the transitional period by ammonia (not shown).

DISCUSSION

The transitional period is when fingers and migrating slugs assess their environment to determine the appropriateness of that environment for culmination and fruiting body formation. A variety of factors seem to be assessed, as revealed by their influence on the time spent as slugs: temperature (3), humidity (21), direction and intensity of light (18), substrate composition (3), and surface water ionic strength (18, 29). Endogenously produced ammonia is used in the assessment, since its volatility lends itself as a reporter of several of the relevant environmental conditions (23, 31). Previously, we demonstrated that the DhkC phosphorelay is an important signaling pathway in the slug/culmination choice. The DhkC pathway affects the concentration of intracellular and extracellular cAMP by controlling the activity of the cAMP phosphodiesterase RegA in response to ammonia levels (28). The ammonium transporter AmtC causes an inhibition of the DhkC phosphorelay in response to low ammonia levels, thus promoting culmination when environmental conditions are conducive to spore dispersal. Hence, it has been proposed that AmtC functions as a signal transduction sensor of low ammonia levels (16). The results presented herein support the hypothesis that AmtA also regulates the slug/culmination choice by functioning as an activator of the DhkC phosphorelay to promote slug migration when ammonia levels are high, thus inhibiting culmination when environmental conditions are not conducive to spore dispersal. Our findings suggest that AmtA may serve as a sensor of the high ammonia levels and stimulates the phosphorelay in response.

Consistent with this model is the fact that developing *amtA* null cells possessed a shorter than normal transitional period of 1 to 2 h and rarely formed slugs under standard conditions of development. The parental strain under these conditions had a transitional period of 2 to 3 h, with substantial numbers of slugs forming and migrating briefly prior to their culmination. The minimal transitional period and formation of few to no slugs observed for the *amtA* null strain is reminiscent of similar behavior that was previously observed for the *dhkC* null strain (28). In the absence of DhkC, the phosphorelay cannot be activated to inhibit culmination and to promote slug formation. Hence, there is a minimal transitional period and a lack of slug formation, as with the *amtA* null strain. In addition, the *amtA* null strain, again like the *dhkC* null strain (28), was insensitive to the promotion of slug formation and migration by the addition of exogenous ammonia. Finally, the loss of AmtA in the *amtC* null strain rescued the slugger phenotype of the latter strain.

For the *amtC*/*amtA* double null strain, both the inhibition of the DhkC phosphorelay by AmtC in response to low ammonia and the herein-postulated stimulation by AmtA in response to high ammonia would be lacking, perhaps resulting in an "indecisive" state. This seemed to be the case, as the vast majority of double null fingers fell to the substrate, giving an initial field of slugs. However, little to no migration occurred over the next 2 to 4 h as the slugs asynchronously rose and began culmination. Presumably, other environmental cues or signals, either through the DhkC phosphorelay or otherwise, eventually promoted culmination, as the standard conditions used strongly support this choice.

It should be noted that the spatial expression of the *amtA* gene is consistent with the proposed function of regulating the DhkC phosphorelay. Previous work found *amtA* to be expressed in various prestalk cell types in a highly dynamic manner, and its expression overlaps spatially with that of *amtC* and *dhkC* during the transitional period (10, 16). Specifically, all three genes are expressed in the prestalk region during the transitional period, and *amtA* and *amtC* localize to the nascent stalk tube at the initiation of culmination.

While the *amtA* null strain and the *dhkC* null strain share phenotypic aberrations, such as bypassing the slug stage, the strains also show differences. Developing *amtA* cells showed neither the early aggregation nor precocious expression of several aggregation-specific and cell-type-specific genes that occurs in the *dhkC* null strain (28). Cells lacking AmtA, but not those lacking DhkC, had a slightly enhanced growth rate in axenic cultures. While the fruiting bodies formed by *dhkC* null cells were morphologically normal, those derived from *amtA* null cells typically were small, though morphologically normal. These differences suggest that AmtA and the DhkC phosphorelay have additional, nonoverlapping functions independent of their joint role mediating the slug/culmination choice.

Finally, disruption of *amtA* within this strain did not rescue certain defects seen in prestalk gene expression within developing *amtC* null cells. An initial delay in *ecmAO* expression

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was seen in both strains. However, normal levels of expression in the double null strain were restored as the slugs began transitioning to culminants and were maintained throughout culmination. In contrast, normal levels of *ecmAO* in *amtC* null were not observed until after several hours of slug migration and then only transiently, as expression was subsequently lost. Interestingly, a similar pattern of reduced expression followed by recovery to normal levels was seen for CudA expression in the double null strain. The lack of correct prestalk gene expression initially followed by normal expression at the onset of culmination suggests that, without either of the Amt inputs into the DhkC phosphorelay, cAMP levels initially are atypical but are corrected upon input from other environmental signals that promote culmination. Alternatively, the effect of the ammonia transporters on early prestalk gene expression may be independent of the DhkC phosphorelay.

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