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-
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 because STATa nuclear localization does not occur (16). CudA (culmination deficient) 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. Dicyostelium 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 developmental timing was similar to that of the wild type, but aggregates and fingers were smaller than normal. Little time did form did not migrate but immediately rose to become 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 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 starved and placed under standard conditions that promote slug formation (not shown). Cullination 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).

RESULTS

Disruption of amtA results in subtle abnormalities. While disruption of the amtA 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 starved and placed under standard conditions that promote slug formation (not shown). Cullination 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).
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.

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 cutA*/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...
both the strains but, curiously, also declines during slug migration in from a STATa-independent mechanism, was seen in all three (3A). The presence of CudA in prespore cells, which results

amtC

double null strain began culmination was in contrast to the contrast, slugs of the erably reduced levels relative to that of the wild type. In con-

amtC

localization of STATa was present (Fig. 4) but at consid-

significant loss of CudA expression during slug migration, nu-

localization of STATa (Fig. 4) at any time and thus no STATa-

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. 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.

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

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-galacto-

sidase activity. Developmental stages and strains are as labeled. Areas of black indicate expression of the ecmAO promoter.
3 h to 6 to 7 h. In contrast, the Fig. 6, ammonia prolonged the slug stage for the Ax4 cells, with absence of exogenously added ammonia. The \( \text{amtC} \) functions as an activator of the DhkC phosphorelay to promote slug formation (16). The results presented herein support the hypothesis that \( \text{AmtA} \) also regulates the slug/culmination choice by functioning as a signal transduction sensor of low ammonia levels (28). The \( \text{dhkC} \) null strain also was insensitive to prolongation of the transitional period by ammonia (not shown).

Fig. 6. Exposure of developing \( \text{amtA} \) 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.

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 in the transitional period increasing from 2 to 3 h to 6 to 7 h. In contrast, the \( \text{amtA} \) null strain had a transitional period of 1 to 2 h independent of the presence or absence of exogenously added ammonia. The \( \text{amtC} \) null/\( \text{amtA} \) null strain also was insensitive to prolongation of the transitional period by ammonia (not shown).

铵离子增强了 slug形成，对铵离子的持续存在显著地延长了Ax4细胞的slug阶段，与存在外源铵离子时的情况形成对照。在铵离子不存在的情况下，\( \text{amtA} \) null突变株的过渡期为1到2小时，独立于铵离子的存在或不存在。\( \text{amtC} \) null/\( \text{amtA} \) null突变株也对铵离子的过渡期延长不敏感（不显示）。

铵离子的过渡期是评估手指和迁移slug评估其环境以确定该环境是否适合成体和子实体形成的时间。评估了多种因素，如温度（3）、湿度（21）、光的方向和强度（18）、底物组成（3）和表面水的电导率（18, 29）。内源生成的铵离子被评估，因为它代表了几个相关环境条件（23, 31）。我们证明了DhkC磷酸二酯酶是触发slug/成体选择的信号通路的重要信号通路。DhkC磷酸二酯酶途径影响了胞内和胞外cAMP的浓度，通过控制cAMP磷酸二酯酶反应来对铵离子水平做出反应（28）。铵离子运输体\( \text{AmtC} \)通过氨基抑制DhkC磷酸二酯酶来阻止低铵离子水平的成体化，从而促进孢子的散播。尽管铵离子的过渡期在低铵离子水平上，促进了成体化，但过渡期的环境条件是诱致的，不利于孢子的散播。因此，它被提议为氨基作为信号转导传感器对低铵离子水平的作用（16）。这些结果支持的假设是\( \text{AmtA} \)也通过作为DhkC磷酸二酯酶的活性物来调节slug/成体选择。

铵离子的过渡期为2到3小时，显著地降低了形成和迁移的slug数量，之前观察到的\( \text{dhkC} \) null突变株的相似行为。在缺乏DhkC的情况下，磷酸二酯酶无法被激活以抑制成体化并促进slug形成。因此，有一个在氨离子水平低下的过渡期，导致低氨离子情况下；漫延 slug形成。氨离子的过渡期对氨离子水平的高可能导致抑制成体化，因此抑制孢子的散播。我们发现\( \text{AmtA} \)可以作为高氨离子水平的传感器，刺激磷酸二酯酶反应。

一致与这个模型的事实是发展\( \text{amtA} \) null突变株比正常过渡期短，通常形成.slug而标准条件下的Ax4细胞。 parental strain under these conditions had a 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 \( \text{amtA} \) null strain is reminiscent of similar behavior that was previously observed for the \( \text{dhkC} \) 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 \( \text{ amtA} \) null strain. In addition, the \( \text{amtA} \) null strain, again like the \( \text{dhkC} \) null strain (28), was insensitive to the promotion of slug formation and migration by the addition of exogenous ammonia. Finally, the loss of \( \text{AmtA} \) in the \( \text{amtC} \) null strain rescued the slugger phenotype of the latter strain.

For the \( \text{amtC}/\text{amtA} \) double null strain, both the inhibition of the DhkC phosphorelay by \( \text{AmtC} \) in response to low ammonia and the herein-postulated stimulation by \( \text{AmtA} \) in response to high ammonia would be lacking, perhaps resulting in an “indeterminate” 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 \( \text{amtA} \) gene is consistent with the proposed function of regulating the DhkC phosphorelay. Previous work found \( \text{amtA} \) to be expressed in various prestalk cell types in a highly dynamic manner, and its expression overlaps spatially with that of \( \text{dhkC} \) and \( \text{dhkC} \) during the transitional period (10, 16). Specifically, all three genes are expressed in the prestalk region during the transitional period, and \( \text{amtC} \) and \( \text{amtA} \) localize to the nascent stalk tube at the initiation of culmination.

While the \( \text{amtA} \) null strain and the \( \text{dhkC} \) null strain share phenotypic aberrations, such as bypassing the slug stage, the strains also show differences. Developing \( \text{amtA} \) cells showed neither the early aggregation nor precocious expression of several aggregation-specific and cell-type-specific genes that occurs in the \( \text{dhkC} \) 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 \( \text{dhkC} \) null cells were morphologically normal, those derived from \( \text{amtA} \) null cells typically were small, though morphologically normal. These differences suggest that \( \text{AmtA} \) and the DhkC phosphorelay have additional, nonoverlapping functions independent of their joint role mediating the slug/culmination choice.

Finally, disruption of \( \text{amtA} \) within this strain did not rescue certain defects seen in prestalk gene expression within developing \( \text{amtC} \) null cells. An initial delay in \( \text{ecmAO} \) expression
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 for the double null strain. The lack of correct prestalk gene expression may be independent of the DhkC phosphorelay.

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