

Regulation of Ammonia Homeostasis by the Ammonium Transporter AmtA in *Dictyostelium*

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Abstract

Ammonia has been shown to function as a morphogen at multiple steps during development of the cellular slime mold *Dictyostelium discoideum*; however, it is largely unknown how intracellular ammonia levels are controlled. In the *Dictyostelium* genome, there are five genes that encode putative ammonium transporters, *amtA*, *amtB*, *amtC*, *rhgA*, and *rhgB*. Here, we show that AmtA regulates ammonia homeostasis during growth and development. We found that cells lacking *amtA* had increased levels of ammonia/ammonium, whereas their extracellular ammonia/ammonium levels were highly decreased. These results suggest that AmtA mediates excretion of ammonium. Supporting a role for AmtA in ammonia homeostasis, AmtA mRNA is expressed throughout the life cycle, and its expression level increases during development. Importantly, AmtA-mediated ammonia homeostasis is critical for many developmental processes. *amtA*⁻ cells are more sensitive to NH₄Cl in chemotaxis towards cAMP and in formation of multicellular aggregates. Furthermore, even in the absence of exogenously added ammonia, we found that *amtA*⁻ cells produced many small fruiting bodies and viability and germination of *amtA*⁻ spores were dramatically compromised. Taken together, our data clearly demonstrate that AmtA regulates ammonia homeostasis and plays important roles in multiple developmental processes in *Dictyostelium*.

Introduction

The cellular slime mold *Dictyostelium discoideum* has a unique life cycle consisting of a unicellular growth phase and a multicellular developmental phase. When food sources such as bacteria are available, *Dictyostelium* amoeboid cells proliferate by cytokinesis. Starvation triggers cells to undergo development processes, during which up to 10^5 cells display chemotaxis towards cyclic AMP and form multicellular aggregates. On top of the aggregates, a small projection is formed, and this process is called tip-formation. Cells located at the tips of aggregates differentiate into prestalk cells, precursors of stalk cells, while the rest of the aggregates become prespore cells, precursors of spores. The tipped aggregates form elongated multicellular structures called slugs. Slugs migrate and eventually culminate to form a fruiting body consisting of a mass of spores supported by a stalk (24, 42, 45).

A number of diffusible molecules regulate the development of *Dictyostelium*, including cAMP, differentiation-inducing factor (DIF), adenosine, and ammonia (5, 45, 72, 76). Ammonia has been shown to affect many developmental events in *Dictyostelium*. For example, in the presence of ammonia, the production and secretion of cAMP are inhibited, resulting in impairment of chemotaxis towards cAMP and subsequent tip-formation during early development (22, 59, 75). At later stages of development, ammonia acts against DIF, suppresses differentiation into prestalk cells, and promotes differentiation into prespore cells (6, 25, 62, 70). In addition, ammonia plays an important role in the choice between forming a migrating slug and culmination. High concentrations of ammonia keep slugs migrating and block the initiation of culmination (58). Exhaustion of ammonia triggers culmination by activating PKA through the DhkC signaling pathway (29, 59, 61). In fruiting bodies, extremely high concentrations of ammonium phosphate in sori maintain spore dormancy through activation of the sporulation-specific adenylyl cyclase ACG (8).

Ammonia is produced by protein catabolism, and ammonia levels rise during development when most energy is generated by degradation of protein and RNA (26, 58, 69, 74). It has been suggested that glutamine synthetases, which incorporate ammonia into glutamine, control intracellular levels of ammonia. The expression of glutamine synthetases is developmentally regulated, and their activity becomes elevated during the culmination stage (12-14, 23), suggesting a role of glutamine synthetases in culmination. Indeed, pharmacological inhibition of glutamine synthetase blocks culmination during development (14). In addition, there are five

genes, *amtA*, *amtB*, *amtC*, *rhgA*, and *rhgB*, which belong to the evolutionarily conserved family of ammonium transporter/methylammonium permease/Rhesus protein (Amt/Mep/Rh) in the *Dictyostelium* genome (15). Previous studies have shown that developmental phenotypes in cells lacking AmtC can be rescued by deleting the AmtA (60). These studies suggest that AmtA and AmtC antagonistically regulate developmental processes, and that the ammonium transporters AmtA and AmtC function in either ammonium transport or ammonium sensing (20, 35, 60). In this study, we show that AmtA regulates intracellular ammonium /ammonia levels during growth and development, and is critical for morphogenesis of multicellular aggregates and normal spore formation.

Materials and Methods

Strains, culture, and development

The *D. discoideum* strain Ax2 (38, 71) was used as wild-type cells in this study. For sequencing, the strain Ax4 (38) was used. Cells were cultured at 22°C either in HL-5 medium (71) or on A-medium agar plates (0.5% Glucose, 0.05% Yeast extract, 0.75 g of Proteose peptone, 16.5 mM KH₂PO₄, 4.0 mM K₂HPO₄, 2.0 mM MgSO₄, 1.5% agar) associated with *Klebsiella aerogenes*. For selection and maintenance of transformants, the medium was supplemented with 10 µg/ml of blasticidin-S (Wako) or 20 µg/ml geneticin (Calbiochem). Peptone and yeast extract were obtained from Difco Laboratories.

For synchronous development, exponentially growing cells were harvested and washed three times in LPS buffer (20 mM KCl, 0.24 mM MgCl₂, 40 mM K₂HPO₄/KH₂PO₄, pH 6.4) and placed on ø 47 mm cellulose membrane filters (Toyo Roshi Kaisha, Ltd.) at a density of 5 x 10⁶ cells/cm². The filters were placed on M-085 paper pads (Toyo Roshi Kaisha, Ltd.) containing LPS and housed in 6 cm-diameter Petri dishes at 22°C. When NH₄Cl was added, ammonia buffer A (20 mM KCl, 2.5 mM MgCl₂, 40 mM KH₂PO₄, pH 7.3) was used instead of LPS buffer. Developmental processes were observed with an Olympus SZX12 microscope. Stalk lengths were determined using the NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

Nucleic acid analyses

Genomic DNA was extracted as described (32, 33). Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Poly (A)-containing RNA was purified using oligotex-dT30 Super (JSR). RNA from prespore and prestalk cells were extracted as previously described (51, 54). Southern and Northern blot analyses were performed as previously described (55). A probe specific to *amtA* was prepared by random priming for a 1.1 kb Eco T22I/Not I-digested fragment of cDNA SSB490.

The *amtA* DNA sequence was determined by sequencing cDNA clones SSB490, SSB126, and SLB420, generated by the Japanese cDNA project (49, 65, 66), and the corresponding region of genomic DNA.

Phylogenetic analysis

A NJ-tree was generated by Clustal program (63) based on pfam database alignment (PF00909). The SwissProt/TrEMBL identifier numbers for the sequences: Dd.AmtA; Q9BLG4, Dd.AmtB; Q9BLG3, Dd.AmtC; Q8MXY0, Mt.MTH661; O26757, Ec.AmtB; P37905, Sc.Mep1p; P40260, Sc.Mep2p; P41948, Sc.Mep3p; P53390, Ce.C05E11.4; P54145, AtAMT1;1; P54144, AtAMT1;2; Q9ZPJ8, AtAMT2;1; Q9M6N7, Cr.Rhp; Q94CJ2, Ce.Rhp-1; Q9N2M5, Dm.Rhp; Q9V3T3, Xl.RhAG; Q9DGD8, Hs.RhAG; O43514, Hs.RhBG; Q9H310, Hs.RhCG; Q9UBD6, Hs.RhCE; P18577, Hs.RhD; Q02161. Amt alpha and beta grouping were based on previous work (37).

Gene disruption and expression

For *amtA* disruption, blasticidin resistance gene (*bsr*) from pBsr Δ Bam (1) was used as a selection marker. The *amtA* coding region in the cDNA SSB490 was amplified by PCR using primers LA-XbaI (5'-gctctagaTGTAACCAACCACCACCCCAAATCC-3') and LA-HindIII (5'-cccaagcttGGTTTGGTTTAAATGCAGGTAGTGC-3'), then the PCR product was ligated with a 1.3 kb XbaI/HindIII fragment of pBsr Δ Bam. For efficient homologous recombination, part of the 5' region of the construct was replaced by genomic sequence containing the first intron. The sequence for displacement was amplified from genomic DNA with the primers LAP-1 (5'-AATCAAGTAGCACCAGATCCAGG-3') and LA-XbaI. The *bsr* gene was oriented in the same direction as the *amtA* gene to ensure the presence of a transcriptional terminator in the middle of the *amtA*-coding sequence. From the resulting vector pAMTAbsr (5.9 kb), the 2.9 kb *amtA/bsr* fragment was excised with SalI and NotI and used for transformation.

To express wild-type *amtA*, a plasmid containing *amtA* was constructed by insertion of a PCR fragment containing the complete *amtA* ORF into the *Dictyostelium* extrachromosomal expression vector HK12neo, a derivative of MB12neo (41).

Determinations of ammonia/ammonium

The intercellular amounts of ammonia were measured as follows (2, 8). Cells were developed on nitrocellulose filters at 5×10^6 cells/cm². Filters were placed on a paper pad containing 2 ml of KK2 buffer (20 mM KH₂PO₄/K₂HPO₄, pH 6.4) and housed in Petri dishes at 22°C. Then cells were harvested in 30 ml of ice-cold KK2 containing 20 mM EDTA at different time points during development, washed and resuspended in 1 ml of KK2 buffer. Cell suspension was

homogenized with a Mini-BeadBeater™ (Wakenyaku Co., LTD.) using 0.5 mm-diameter glass beads at 5,000 rpm for 3 min. For extracellular measurements, the paper pad under the nitrocellulose filter were soaked in 20 ml of KK2 buffer for 20 min to allow the ammonia to diffuse from the pad, and the resultant solution was used as a sample of the extracellular environment of the cells. To measure ammonia in spores and spore mass solution, cells were developed on membrane filters, and sori were collected using a glass capillary. After centrifugation at 1,400 g for 5 min, the supernatant was used as spore mass solution. The pellet of spores was resuspended in 1 ml of KK2, and then homogenized as described above. The concentrations of ammonia/ammonium in sample solutions were measured, using an ammonia electrode Ti-9001Ka (Toko Chemical Laboratories Co., Ltd.), following manufacturer's instructions.

Spore viability and germination

Germination rates of spores were determined as described (48). Spores were collected in KK2 buffer from fruiting bodies on agar plates by a wire loop. To induce synchronous germination, 30 µl of supernatant from an overnight culture of *K. aerogenes* in A-medium was added to 0.3 ml of spore suspension in a siliconized 20 ml test tube (27). After shaking for at 150 rpm at 22°C for 6 hrs, germinated spores were counted using a hemocytometer. To determine the viability of spores, vital staining with propidium iodide (PI) was used (48). Spores were collected at 2, 4, 6, and 8 days after fruiting body formation, suspended in KK2 buffer, and stained with 20 µg/ml of PI (Sigma) for 5 min.

Glucose Assay

Glucose levels were measured as described (81) with a minor modification. Cells were grown at 22°C in HL-5 medium with maltose as a carbohydrate source in shaking cultures. Cells were harvested, developed for 0, 6 and 12 hrs in shaking cultures and collected by centrifugation. The pellets were lysed by freezing at -70 °C for overnight. The pellets were thawed and resuspended in distilled water at 6×10^8 cells/ml. The lysates were clarified by centrifugation at 12,000 g for 5 min, and 80 µl of the supernatants was used to measure glucose using a Sigma glucose assay kit (GAGO20) following manufacture's instruction. Protein concentration of lysate was determined using the Bio-Rad protein assay kit (500-0006).

Immunoblotting

Cells were developed on agar plates, and spores were harvested. Whole cell lysates were subjected to SDS-PAGE and blotted on PVDF membranes. Blots were immunostained with monoclonal anti-actin antibody (C4, Chemicon) or monoclonal anti-phosphotyrosine antibody clone PY20 (ICN), as previously described (79).

Results

AmtA controls ammonia homeostasis during growth and development

The *Dictyostelium* genome contains five genes, *amtA*, *amtB*, *amtC*, *rhgA*, and *rhgB*, which belong to the ammonium transporter/methylammonium permease/Rhesus protein family (Amt/Mep/Rh). A phylogenetic analysis showed that these five genes are divided into three groups; Amt alpha, Amt beta and Rh (Fig. 1A). Although most organisms carry only one type of Amt/Mep/Rh gene (for example, vertebrates only carry genes belong to the Rh group), *Dictyostelium* has all three types. In *Dictyostelium*, there are two genes that belong to each of the Rh (*rhgA* and *rhgB*) and Amt beta (*amtB* and *amtC*) groups, and the Amt alpha group contains a single gene, *amtA*. In this paper, we focused on the characterization of *amtA*. *amtA* encodes a 463 amino acid protein (49.1 kDa), and a hydropathy analysis suggests that AmtA contains 11 transmembrane segments. AmtA protein displays 11.0-23.6% identity to other Amt/Mep/Rh proteins in *Dictyostelium*, and 23.8-35.6 % identity to proteins in the Amt alpha group in other organisms.

To determine whether AmtA regulates ammonium homeostasis, we disrupted the *amtA* gene in *Dictyostelium* by homologous recombination. The disruption was confirmed by Southern blot analysis (Fig. 1B, C). Wild-type cells showed 5.1 and 3.5 kb DNA fragments generated by HincII-digestion and by BamHI/EcoRV-digestion, respectively. On the other hand, 6.2 and 4.6 kb fragments were observed in cells disrupted for the *amtA* gene. We measured levels of intracellular and extracellular ammonia/ammonium during growth and development using ammonia-specific electrodes (2, 8). We observed 5.24 ± 0.14 fmol/cell ammonia/ammonium in wild-type cells during growth (Fig. 2A). In contrast, in *amtA*⁻ cells, intracellular ammonium/ammonia levels were significantly increased. The mutants contained 8.0 ± 0.7 fmol/cell ammonium/ammonia, showing an approximate 1.5-fold increase. When we induced development by starvation, intracellular levels of ammonium/ammonia gradually decreased in wild-type cells during development. Although *amtA*⁻ cells also showed a gradual decrease in ammonia/ammonium levels, intracellular levels of ammonia/ammonium in *amtA*⁻ cells were much higher than those seen in wild-type cells at both the aggregation stage (12 hr after starvation) and culmination stage (20 hr). Increases in intracellular ammonia/ammonium levels could result from either overproduction of ammonia/ammonium or defects in its transport out of cells. Supporting the latter possibility, we found that extracellular levels of

ammonium/ammonia were decreased in *amtA*⁻ cells (Fig. 2B). We confirmed that these phenotypes were caused by loss of the *amtA* gene by expressing wild-type *amtA* in *amtA*⁻ cells. All the phenotypes were significantly rescued by the expression of AmtA. These results clearly demonstrate that AmtA is required for normal ammonia homeostasis during growth and development.

Consistent with a role of AmtA in ammonia homeostasis, we found that *amtA* 1.7 kb mRNA is expressed during both growth and development using Northern blot analysis (Fig. 3A). The expression level increased continuously during development. This is in contrast with previous studies showing that *amtA* mRNA levels do not change during development using RT-PCR (20). The apparent difference may result from different methods used to measure mRNA levels. Nevertheless, the previous study and our current study clearly demonstrate that AmtA is expressed during development. Furthermore, we confirmed the localization of *amtA* mRNA in prespore cells in slugs (20) using Northern blotting (Fig. 3B).

We confirmed previous studies (60) that *amtA*⁻ cells normally grow in both shaking culture and on bacterial plates (data not shown), that wild-type and *amtA*⁻ cells show similar sensitivities to ammonia in culmination (Fig. 3C), and that the time-course of development in wild-type and *amtA*⁻ cells are similar, differentiating into fruiting bodies in 24 hr upon starvation (data not shown). In Fig 3C, cells were developed in the absence of NH₄Cl, and then different amounts of NH₄Cl were added to pads for further development. In addition, *amtA*⁻ cells produced more aggregates and fruiting bodies than wild-type cells (Fig. 3D, E and F). In wild-type cells, the average density of aggregates was 593 aggregates/cm². In contrast, *amtA*⁻ cells produced 1128 aggregates/cm². Furthermore, the fruiting bodies of *amtA*⁻ cells are shorter than those of wild-type cells. The average stalk length in *amtA*⁻ (0.77 mm) was almost half of wild-type length (1.63 mm). Expression of wild-type AmtA in *amtA*⁻ cells significantly suppressed those *amtA*⁻ phenotypes. These results are consistent with the previous observation that AmtA plays a critical role in the number and size of aggregates and fruiting bodies (60). We also found that *amtA*⁻ cells show smaller aggregation territory sizes and frequent group breakup (Fig. 3G). Since glucose has been suggested to increase aggregation size, we measured intracellular levels of glucose (81, 82). We found that glucose levels in *amtA*⁻ cells are higher than those in wild-type cells (Fig. 3H). In contrast, protein amounts in wild-type and *amtA*⁻ cells are indistinguishable (data not shown). These results suggest that the decrease of aggregation sizes in *amtA*⁻ cells are mainly affected by ammonia, but not glucose.

***amtA*⁻ cells are hypersensitive to ammonia in early morphogenesis**

It has been shown that NH₄Cl inhibits multiple steps in early developmental processes including aggregation, tip-formation of aggregates (10, 22, 58). Since *amtA*⁻ cells contain higher levels of ammonia/ammonium in early development, we reasoned the mutant cells may be more sensitive to ammonia than wild-type cells. To test this idea, we induced development in *amtA*⁻ and wild-type cells in the presence of ammonia. Cells were incubated on filter membranes containing 0, 30, or 50 mM of NH₄Cl throughout development. As shown in Fig. 4A, in wild-type cells, ammonia inhibited aggregation and tip-formation in a concentration-dependent manner. In the absence of NH₄Cl, wild-type cells developed normally and formed fruiting bodies in 36 hr after starvation. However, in the presence of 30 mM NH₄Cl, wild-type cells were only able to form aggregates with pointed ends (tip-formation) and did not differentiate further into fruiting bodies. At 50 mM, wild-type cells aggregated without tip-formation. This result is not simply due to a delay in development. When we examined cells for prolonged periods of time (3 days), the cells still did not form fruiting bodies. In contrast, the mutants did not show tip-formation of aggregates at 30 mM NH₄Cl, although *amtA*⁻ cells formed fruiting bodies in the absence of NH₄Cl. Furthermore, 50 mM NH₄Cl completely blocked aggregation in *amtA*⁻ cells. These results show that loss of AmtA makes cells hypersensitive to ammonia in aggregation and tip-formation during development.

***amtA*⁻ cells are defective in chemotaxis in the presence of ammonia**

The inhibitory effect of ammonia on the aggregation of *amtA*⁻ cells suggests that ammonia suppresses chemotaxis towards cAMP in the mutant cells. To test this possibility, we examined chemotaxis towards cAMP using a spot assay (Fig. 4B). Cells were spotted on the center of plates that contained 10 μM cAMP in the presence or absence of NH₄Cl. Since there are no nutrients available on the plate, cells initiated development processes immediately after being spotted. Cells secrete phosphodiesterase and degrade extracellular cAMP, generating a difference in cAMP concentration around the spot with higher concentrations outside and lower concentrations inside. Along the cAMP gradient, cells move outward from the spotted area. If cells are defective in chemotaxis, they would stay inside the spotted areas. When wild-type and *amtA*⁻ cells were spotted on plates containing 10 μM cAMP in the absence of NH₄Cl, both wild-type and mutant cells developed normally. At the periphery of the spotted area, we found

that many cells moved outward (Fig. 4B, arrows). This outward movement is chemotactic migration towards cAMP since cells move out only when cAMP is present. On plates lacking cAMP, cells developed normally, but they stayed in the spotted area and did not move outward. However, when we added 30 mM NH₄Cl to the plates, wild-type and *amtA*⁻ cells showed distinct chemotactic behaviors. In the presence of 30 mM NH₄Cl, wild-type cells were still able to develop, form aggregates, and move outward towards cAMP. In contrast, when *amtA*⁻ cells were spotted, the mutant cells developed normally and formed aggregates, but they failed to move out of the spotted area. Our data suggests that cells lacking AmtA become more sensitive to NH₄Cl during chemotaxis towards cAMP.

AmtA is required for normal spore formation

In *Dictyostelium*, spore formation process affected by ammonia through regulation of cAMP-dependent protein kinase (PKA) (29, 78). In addition, extra spore solution contains high concentrations of ammonium phosphate, which has been suggested to maintain dormancy of spores (8, 9, 68). To determine whether AmtA is involved in ammonium homeostasis in spore and extra spore solution in sori, we collected sori and separated spores from extra spore solution by centrifugation, and measured levels of ammonia/ammonium. As shown in Fig. 5A, levels of ammonia/ammonium are increased by two-fold in *amtA*⁻ spores. Wild-type spores contained 1.50 fmol/cell ammonia/ammonium, whereas *amtA*⁻ spores contained 2.69 fmol/cell ammonia/ammonium. In addition, ammonia levels in extra spore solution decreased two-fold in the mutant cells (Fig. 5B). Therefore, our data indicate that AmtA is required for normal ammonia homeostasis in spore.

To probe the role of ammonia in spores, we examined the morphology, viability, and germination rate of spores. We observed spore morphology using phase contrast microscopy and checked their viability using propidium iodine (PI), which only stains dead spores (7, 48, 50). We found that spores lacking AmtA are compromised in viability as they age. When we collected spores immediately after the completion of fruiting body formation, both wild-type and mutant spores showed oval shapes, and very few spores were stained by PI. However, as spores aged, *amtA*⁻ spores appeared to be darker under phase contrast microscope (Fig. 5C, D). The majority of the dark spores were stained by PI, indicating that they were dead. Twelve days after fruiting body formation, 79.0% of *amtA*⁻ spores were stained by PI, whereas only 4.4% of wild-type spores were PI-positive. Furthermore, we found that germination rates were also

decreased in *amtA*⁻ spores (Fig. 5E). Probably as the result of their compromised viability, *amtA*⁻ spores are also defective in normal germination. Immediately after spore formation, ~95% of wild-type spores germinated whereas only 67% of *amtA*⁻ spores germinated. Eight days after completion of fruiting body formation, wild-type cells maintained germination rates similar to that seen at 0 day. In contrast, the germination rate of *amtA*⁻ spores became decreased as they aged. We found that only 20% of *amtA*⁻ spores germinated at 8 day. Supporting the idea that *amtA*⁻ cells are defective in normal spore formation, we found that tyrosine-phosphorylated actin levels decreased in *amtA*⁻ spores (Fig. 5F). It has been shown that actins are organized into thick bundles in spores and are phosphorylated on tyrosine residues during the formation and maturation of spores (36, 57). Therefore, our data strongly suggest that AmtA is required for the formation and maintenance of viability of spores.

Discussion

A large number of studies have shown that ammonia regulates multiple processes during development of *Dictyostelium* (43, 45, 76-78). However, molecular mechanisms underlying ammonia homeostasis are poorly understood. Previous studies have suggested that AmtA functions in ammonia transport or ammonia sensing (20, 60). In this paper, supporting a role in ammonium transport, we have shown that AmtA is required for normal ammonia homeostasis in *Dictyostelium* during growth and development. Our data strongly suggest that AmtA is involved in the excretion of ammonia/ammonium from cells. Supporting our conclusion, cells lacking AmtA accumulate ammonia/ammonium. The impairment of ammonium efflux results in reduction in extracellular levels of ammonia/ammonium. Furthermore, intra-spore levels of ammonia/ammonium are also highly increased in *amtA*⁻ mutants. Since extracellular levels of ammonia/ammonium are slightly higher than intracellular levels in *Dictyostelium*, AmtA might actively transport ammonium out of cells. Although our data suggest that AmtA is an important ammonium transporter in development, they do not rule out the possibility that AmtA also functions in ammonia sensing as previously proposed (60).

Previous studies have shown that AmtA is important for morphogenesis of fruiting bodies and for resistance to high concentrations of ammonia in culmination (60). Our study confirmed the previous findings and further identified three additional developmental processes that involve AmtA-mediated ammonia homeostasis, including chemotaxis, tip-formation and spore formation. At the beginning of development, single cells undergo chemotaxis towards aggregation centers, which release the chemoattractant cAMP, leading to formation of multicellular aggregates. We found that AmtA regulates the sensitivity to ammonia in chemotactic migration towards cAMP. *amtA*⁻ cells fail to undergo chemotaxis in the presence of NH₄Cl. Since the concentrations of NH₄Cl that block chemotaxis do not inhibit normal development of *amtA*⁻ cells, it is unlikely that the chemotaxis phenotypes simply result from developmental defects. In addition, in our experiments, we examined chemotaxis towards exogenously added cAMP. Therefore, the observed chemotaxis defect is not due to inhibition of the production and secretion of cAMP by ammonia. Rather, we suggest that ammonia directly affects signaling pathways for chemotaxis. Supporting our hypothesis, it has been shown that ammonia affects intracellular pH and thereby inhibits chemotaxis (10, 19, 67). It is possible that intracellular pH is regulated at least partially by AmtA during chemotaxis. Since the morphogenesis of aggregates involves cAMP signaling

(64), the impaired chemotaxis may result in defects in tip-formation in aggregates in *amtA*⁻ cells. Furthermore, at the final stage of development, *amtA*⁻ cells formed much smaller fruiting bodies than wild-type cells even in the absence of exogenously added ammonia. Many smaller fruiting bodies may result from incomplete chemotaxis, which could produce many smaller aggregates. Alternatively, it is also possible that ammonia homeostasis directly controls the size of the fruiting bodies by affecting differentiation of spore and stalk cells.

One of our most interesting observations is that AmtA regulates ammonia levels in spores. We found that *amtA*⁻ spores in fruiting bodies contained higher levels of ammonia/ammonium whereas extracellular levels were decreased. In addition, *amtA*⁻ spores were severely defective in viability and these phenotypes became more severe as spores aged. It has been shown that ammonia in sori is critical for formation and maintenance of spore (8, 9, 29, 68, 78). Thus, our studies demonstrate that AmtA is a critical regulator for ammonia homeostasis during spore formation. It is likely that the reduced viability of *amtA*⁻ spores leads to their defect in germination. In contrast to cells lacking the histidine kinase *dhkB*, which are defective in spore dormancy and prematurely germinate from spores (80), we did not observe premature germination in *amtA*⁻ spores, it is unlikely that *amtA*⁻ cells are unable to maintain spore dormancy. It has been suggested that the organization of actin cytoskeleton is important for the formation and stabilization of spores (36, 56, 57). In spores, a large fraction of actin molecules are phosphorylated on tyrosine residues and organized into thick bundles. This process is induced under PKA activity, and later, mediated by the MADS-box transcription factor SrfA (16-18). We found that tyrosine phosphorylation of actin is greatly reduced in *amtA*⁻ spores, suggesting that ammonia homeostasis might regulate spore formation and stabilization through affecting actin organization mediated by PKA and SrfA.

Our current study and previous study have shown that disruption of AmtA does not cause severe defects in the culmination step of aggregates on its own (60). Wild-type and *amtA*⁻ cells showed a similar sensitivity to exogenously added ammonia in culmination, suggesting that other ammonium transporters regulate ammonia levels at the initiation of culmination. Supporting this idea, previous studies showed that AmtC was important for induction of culmination and that cells lacking AmtC failed to culminate and remained as slugs (20, 35). In contrast to AmtA, AmtC is proposed to function as a sensor which monitors ammonia levels and activates intracellular signaling. Consistent with this idea, AmtC is preferentially expressed at the tip region of slugs, where culmination signal may be generated (20). Interestingly, the disruption

of *amtA* in *amtC* null strain restored its ability for cells to differentiate to undergo culmination stage (60). In this study, we showed that *amtA* functions as an ammonium transporter, although it is possible that AmtA controls the slug/culmination transition as an ammonia sensor. To determine whether AmtC functions as an ammonium sensor or transporter, it would be important to determine whether intracellular levels of ammonia are also altered in *amtC* cells.

Members of the Amt/Mep/Rh family are found in all domains of life and involved in a variety of biological processes. In microorganisms, ammonia is a nutrient, and most Amt/Mep/Rh proteins participate in uptake of ammonium into cells (11, 30, 34, 40, 47). In contrast, in animals, ammonia is a waste product resulting from amino acid catabolism (28). Rh proteins have been suggested to excrete ammonia to maintain intracellular homeostasis (3, 4, 31, 46, 53, 73), although several recent studies suggested that the substrate of Rh proteins is CO₂, and not ammonia (39, 52). Furthermore, yeast Mep2 ammonium permease has been shown to function as a sensor of ammonia, generating a signal to regulate pseudohyphal differentiation (21, 44). The Mep2 may lack the ability to transport ammonium across membranes (44). In contrast to most organisms that carry only one type of ammonium transporters among the three sub-families (Amt alpha, Amt beta, and Rh groups), *Dictyostelium* contains five ammonium transporters that belong to all three sub-families. It would be tempting to speculate that *Dictyostelium* uses different functions of ammonium transporters, such as ammonium uptake, efflux, and sensing, to control their unique development.

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Figure Legends

Fig. 1. Disruption of the *amtA* gene.

(A) Phylogenetic analysis of ammonium transporters in *D. discoideum* (Dd), *M. thermoautotrophicum* (Mt), *E. coli* (Ec), *S. cerevisiae* (Sc), *C. reinhardtii* (Cr), *C. elegans* (Ce), *D. melanogaster* (Dm), *X. laevis* (Xl), *H. sapiens* (Hs), and *A. thaliana* (At). Scale indicates nucleotide substitutions per site. (B) Disruption of *amtA*. A blasticidin resistance marker (*bsr* cassette) replaced the second intron of the *amtA* gene by homologous recombination. Exons are indicated by white boxes. B, Hc, and V indicate BamHI, HincII, and EcoRV restriction sites, respectively. (C) The *amtA* disruption was confirmed by Southern blotting using a probe shown in B. Genomic DNA isolated from wild-type and *amtA*⁻ cells was analyzed after digestion with HincII (Hc), or BamHI and EcoRV (B+V).

Fig. 2. AmtA is required for intracellular ammonia homeostasis.

(A) Intracellular ammonia/ammonium levels. Cells were collected at 0, 12, and 20 hrs of development, homogenized, and amounts of ammonia/ammonium were determined by an ammonia electrode. Values indicate mean ± SEM (n = 3). (B) Extracellular ammonia levels. Media were collected at indicated time points, and amounts of ammonia were determined as described. Values indicate mean ± SEM (n = 3).

Fig. 3. *amtA*⁻ cells display aberrant morphology of fruiting bodies.

(A) Poly (A)-containing RNAs were collected at the indicated time points and analyzed by Northern blotting with *amtA* cDNA as a probe. Five micrograms of Poly (A)-containing RNAs were loaded in each lane. (B) *amtA* mRNA accumulates in prespore cells. Expression in respective cell-types at slug stage. Total RNA were extracted from prespore (psp) and prestalk (pst) cells in slugs. *ecmA* is used as a marker for prespore cells. (C) Effects of ammonia on culmination. Cells were developed in the absence of ammonia for 14 hrs, and then transferred onto paper pads containing the indicated concentrations of NH₄Cl. Photographs were taken 10 hrs after transfer. Bar, 1 mm. (D) *amtA*⁻ cells produce many small fruiting bodies. Wild-type cells (WT), *amtA*⁻ cells, and *amtA*⁻ cells expressing *amtA* were plated on nitrocellulose filters to induce development and observed at 24 hrs. Bar, 0.5 mm. (E) Quantitation of aggregate density. Cells were plated on filters at a density of 5 x 10⁶/cm². After 12 hrs, aggregates in an

area of 81 mm² were counted. Values indicate mean ± SEM (n = 3). (F) Quantitation of stalk length. Values indicate mean ± SEM (n ≥ 50). (G) Wild-type and *amtA*⁻ cells were developed on non-nutrient plates for indicated time. *amtA*⁻ cells show smaller aggregation territory sizes and increased group breakup. Cells were plated at a density of 5 x 10⁵/cm² and 5 x 10⁶/cm². Bar, 1 mm. (H) *amtA*⁻ cells show higher glucose levels. Cells were developed for 0, 6 and 12 hrs, and analyzed for glucose levels. Values indicate mean ± SEM (n ≥ 5).

Fig. 4. Effects of ammonia on tip-formation, chemotaxis and culmination.

(A) Effects of ammonia on aggregation and tip-formation. Wild-type and *amtA*⁻ cells were placed on nitrocellulose filters and allowed to develop for 36 hrs in the presence of 0, 30, 50 mM NH₄Cl. Bar, 0.5 mm. (B) Effects of ammonia on chemotaxis towards cAMP. Wild-type and *amtA*⁻ cells were plated on non-nutrient plates in the presence or absence of 10 μM cAMP and observed after 6 hrs. As indicated, plates contain 0 or 30 mM NH₄Cl. We determined the original position of the edge immediately after cells were spotted. Bar, 0.2 mm.

Fig. 5. AmtA is required for ammonia homeostasis, viability, and germination of spores.

(A and B) Ammonia levels in spores (A) and spore mass solution (B). Three days after fruiting body formation, spore masses were collected, and spores and sori media were separated by centrifugation. Ammonia levels of each fraction were determined by an ammonia electrode. Values are normalized to 10⁸ spores in 10 μl sori in B. (C) Spores were collected at 0 and 12 days after fruiting body formation was completed and stained by PI. Spores were observed by phase contrast and fluorescence microscopy. Bar, 50 μm. (D) Quantitation of PI-positive spores. Spores were collected at the indicated period of time after fruiting body formation and stained with PI. Spores that were stained by PI were counted. (E) Spores were collected at the indicated time points after the completion of fruiting bodies and examined for germination as described in Materials and Methods. (F) Changes of tyrosine phosphorylation of actin with aging. Total proteins were prepared from 1, 2, 6 and 10 days-old spores, and immunostained with anti-actin antibody and anti-phosphotyrosine antibody.