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Ammonium transporter *C* of *Dictyostelium discoideum* is required for correct prestalk gene expression and for regulating the choice between slug migration and culmination

Janet H. Kirsten, Yanhua Xiong, Andrew J. Dunbar, Meena Rai, Charles K. Singleton *

Department of Biological Sciences, Vanderbilt University, VU Station B 351634, Nashville, TN 37235-1634, USA

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Abstract

Ammonium transporter *C* (*AmtC*) is one of three transporters in *Dictyostelium* that have been proposed to regulate entry and exit of ammonia in a cell type dependent manner and to mediate ammonia signaling. Previous work demonstrated that disruption of the *amtC* gene results in a slugger phenotype in which the cells remain as migrating slugs when they should form fruiting bodies. More detailed studies on the null strain revealed that differentiation of prestalk cell types was delayed and maintenance of prestalk cell gene expression was defective. There was little or no expression of *ecmB*, a marker for the initiation of culmination. Normal expression of *CudA*, a nuclear protein required for culmination, was absent in the anterior prestalk zone. The absence of *CudA* within the tip region was attributable to the lack of nuclear localization of the transcription factor *STATA*, despite expression of adenylyl cyclase A mRNA in the slug tips. Disruption of the histidine kinase gene *dhkC* in the *amtC* null strain restored *STATA* and *CudA* expression and the ability to culminate. The results suggest that the lack of nuclear translocation of *STATA* results from low cAMP due to a misregulated and overactive *DhkC* phosphorelay in the *amtC* null strain.

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Keywords: *Dictyostelium*; Ammonium transporter; *STATA*; *CudA*; Adenylyl cyclase; Histidine kinase; Phosphorelay; Ammonia; *DhkC*

Introduction

Dictyostelium discoideum is a social amoeba that forages on bacteria in the leaf litter and soil of temperate forests in its unicellular state. When food sources are scarce, approximately 100,000 amoeba aggregate into a multicellular structure from which approximately 80% eventually will be dispersed as spores. After several differentiation events, a standing cylindrical structure referred to as a finger is formed. The anterior region is made up of several prestalk cell types and a larger posterior region (ca. 80%) is composed of prespore cells within which a few anterior-like cells (ALCs) that express genes characteristic of prestalk cells are interspersed. A critical decision must be made depending on environmental conditions on whether to initiate culmination of development and form a mature fruiting body or to fall to the substratum and migrate as a

slug seeking more favorable conditions to maximize successful spore dispersal.

Within this paper, the term transitional period refers to the time period as first fingers transition to early culminants, and includes the variable time spent, if any, in the migrating slug stage. Whether the transitional period is brief or includes an extended slug migration phase, culmination entails coordinating several morphological changes that ultimately result in a fruiting body with mature stalk cells and spores. The ability to sense the environment, and hence “decide” when to culminate, is mediated by prestalk cells within the anterior most tip of the finger and slug (Smith and Williams, 1980), a region often referred to as an organizer (Durstun, 1976; Farnsworth, 1973; Raper, 1940).

The initiation of culmination is manifested by the formation of a small cone of prestalk AB cells within the prestalk A region at the anterior of the finger or slug. The terminology references expression of the extracellular matrix proteins, *EcmA* and *EcmB*, the first markers identified that distinguished the various subtypes of prestalk cells and their

* Corresponding author. Fax: +1 615 343 6707.

E-mail address: charles.k.singleton@vanderbilt.edu (C.K. Singleton).

spatial patterning (Jermyn et al., 1989). Conversion of a subset of the prestalk A cells expressing only *EcmA* (prestalk A* cells; Fukuzawa and Williams, 2000) to prestalk AB cells expressing both *EcmA* and *EcmB* indicates the initiation of culmination and can be visualized with a reporter gene driven by the promoter of *ecmB* (Jermyn et al., 1989; Jermyn and Williams, 1991) or the more recently identified *aslA* (acetyl-CoA synthetase-like A) gene (Shimada et al., 2005). Prestalk AB cells produce a nascent stalk tube into which surrounding prestalk A cells are recruited, tip cells first, until the tube elongates downward to reach the substratum (Shimada et al., 2005). The maturing stalk cells within the tube secrete factors that signal to the prespore cells to begin differentiation into mature spores (Anjard et al., 1997, 1998a,b), and the spore mass begins moving up the stalk after it reaches the substratum. Several environmental factors reflect a good or poor environment for dispersal of spores and thereby affect the time spent in the transitional period (Newell et al., 1969; Slifkin and Bonner, 1952). Two major regulators are light and ammonia, a “waste” product produced by the cells via protein degradation to generate energy. *Dictyostelium* cells have co-opted this metabolic by-product as a signaling molecule to mediate several processes during development, including the slug/culmination choice (Follstaedt et al., 2003; Schindler and Sussman, 1977). Low extracellular ammonia promotes culmination while high extracellular concentrations result in slug migration.

Ammonia is thought to mediate the alternative outcomes of slug migration versus culmination via modulation of cAMP dependent protein kinase A (PKA) (Hopper et al., 1993; Schindler and Sussman, 1979; Singleton et al., 1998). A role for activation of PKA in initiating culmination has been demonstrated by several studies (Anjard et al., 1992; Kay, 1989; Mann et al., 1994; Mann et al., 1992), including the finding of a slugger phenotype in which culmination cannot occur when activation of PKA is inhibited (Harwood et al., 1992a,b). Schindler and Sussman (1979) demonstrated that ammonia reduces cAMP production, and expression of a dominant inhibitor of PKA in prespore cells results in hypersensitivity to some of the effects of ammonia (Hopper et al., 1993). Expression of the dominant inhibitor of PKA in prestalk cells results in a slugger phenotype lacking proper gene expression in and differentiation of the prestalk cells (Zhukovskaya et al., 1996).

One regulator of PKA activity is the histidine kinase DhkC, which is thought to regulate the slug/culmination outcome through a phosphorelay that controls the cAMP phosphodiesterase RegA (Singleton et al., 1998). It has been proposed that the DhkC phosphorelay mediates ammonia signaling by coupling ammonia sensing to cAMP concentrations and thus PKA activity. High local ammonia levels are thought to activate the phosphorelay resulting in the activation of RegA and thus inhibiting PKA activity and culmination. When local ammonia levels are low, the phosphorelay is inactive, allowing cAMP accumulation to activate PKA and subsequently initiate culmination (Singleton et al., 1998).

Two other proteins involved in the slug/culmination decision are STATa (signal transducers and activators of transcription) and CudA (culmination deficient). In fingers and slugs, nuclear localization of STATa becomes limited to the prestalk A cells (Araki et al., 1998). Nuclear localization is mediated by binding of extracellular cAMP to the cAMP receptor cAR1 with subsequent phosphorylation of a tyrosine residue of STATa resulting in its dimerization and translocation to the nucleus (Dormann et al., 2001a). Within the nucleus of prestalk A cells, STATa represses the expression of the *ecmB* gene (Mohanty et al., 1999; Shimada et al., 2004) and has been proposed to regulate the commitment to stalk cell differentiation and the initiation of culmination by inhibiting the premature conversion of prestalk A cells to prestalk AB cells (Mohanty et al., 1999). Additionally, STATa is required either for establishing prestalk AB cells or for inducing the majority of genes identified to be specifically expressed in those cells during the transition from slug to culminant (Fukuzawa and Williams, 2000; Shimada et al., 2004).

Within the prestalk A* cells and the cone of prestalk AB cells, STATa induces the expression of the nuclear localized protein CudA (Fukuzawa and Williams, 2000). The molecular function of CudA remains undefined but it is required for culmination as revealed by a slugger phenotype in the *cudA* null strain (Fukuzawa et al., 1997). Thus, while generally inhibiting premature culmination in prestalk A cells, STATa also induces *cudA*, which is required for the conversion of prestalk A* cells into prestalk AB cells to initiate culmination (Araki et al., 1998; Fukuzawa and Williams, 2000). Adenylyl cyclase A (ACA) produces the extracellular cAMP required for STATa nuclear localization and thus indirectly induces CudA expression in the tip cells (Verkerke-van Wijk et al., 2001). Significantly, *acaA* mRNA expression becomes limited to tip cells during late slug migration, and this spatial alteration contributes to differences in cAMP levels within different cell types during the transitional period. The dynamics of these changes may be facilitated by hydrolysis of intracellular cAMP by the activity of the DhkC phosphorelay.

Dictyostelium possesses three ammonium transporters (AmtA, B, and C) that have been proposed to both regulate entry and exit of ammonia from cells in a cell type dependent manner and to mediate ammonia signaling (Follstaedt et al., 2003). We recently showed that *amtC* has an unusual spatial expression pattern similar to that of the *cudA* gene and that like the *cudA* null, disruption of the *amtC* gene results in a slugger phenotype (Follstaedt et al., 2003). Herein, we investigated the role of AmtC during the transition from slug to culminant and found that many of the defects in the *amtC* null strain could be attributed to misregulation of the DhkC phosphorelay.

Materials and methods

Cell growth and development

Strains of *Dictyostelium* were maintained as axenic cultures in HL5 medium (Sussman, 1966) and on SM plates with *Klebsiella pneumoniae* as a bacterial food source (Singleton et al., 1987). Two different *amtC* null strains (BS154 and BS162) derived from Ax4 and with identical phenotypic

characteristics were used interchangeably. BS154 was generated using an interrupting blasticidin cassette as described earlier (Follstaedt et al., 2003) while BS162 was generated using a similar disruption construct with the blasticidin cassette replaced by a hygromycin cassette. The *amtC* gene was disrupted in a *regA*⁻ background (HM1015 (Thomason et al., 1998)) using the disruption construct previously described (Follstaedt et al., 2003) to generate an *amtC/regA* double null strain (BS164). The *dhkC* gene was disrupted in BS162 using the disruption construct previously described (Singleton et al., 1998) to produce the *amtC/dhkC* double null (BS163). All disruptions were confirmed by PCR using one primer within the selection cassette and one primer in the disrupted gene of interest external to the integration site.

Plasmids to express *cuda* with the *actin-15*, *ecmA*, and *pspA* promoters were kindly provided by M. Fukuzawa and were transformed into both *amtC* null strains (BS154 and BS162) by calcium phosphate (De Lozanne and Spudich, 1987; Knecht et al., 1986). Selection was with 10 µg/ml G418.

In preparation for development, 1×10^6 cells were transferred onto SM plates with *K. pneumoniae* and grown at 21°C for 36–48 h. Cells were harvested into PDF (22 mM potassium phosphate, pH 6.5, 20 mM KCl, 5 mM MgCl₂, and 0.5 mg/ml streptomycin sulfate) and centrifuged at low speed to remove bacteria. The *Dictyostelium* cells were suspended in PDF and plated for development on Millipore filters (HABP04700) at 3×10^4 cells/mm² atop cellulose pads soaked in PDF (standard conditions) or on filters atop 1% water agar when Ax4 slug migration was required. Multicellular structures were collected at appropriate times for RNA isolation, in situ hybridization, or immunohistochemical staining.

RT-PCR

RNA was isolated from growing cells and at various times after the initiation of development using Trizol (Sigma) as per the manufacturer's instructions. RT-PCR was carried out as described (Pekovich et al., 1998). In most RT-PCR reactions, *H7*-specific oligonucleotides were used as an internal control as *H7* mRNA is expressed at a constant level during growth and development (Zhang, 1995).

In situ hybridization

In situ hybridization was done as described (Follstaedt et al., 2003) with the following modifications. Riboprobes of 400–600 bp in length were prepared for *acaA*, *amtC*, *cuda*, and *dhkC* using DIG RNA Labeling Mix (Roche 1 277 073) as per the manufacturer's instructions with either T7 or SP6 polymerase and appropriately digested plasmids as templates. Cells were plated on hydrophilic filters (Millipore FHLC 02500) on 1% water agar and developed under light or dark conditions. Structures were harvested at the stages of interest and bound to the filters by transferring to and quickly drying the filter on a sterile cellulose pad for 15–30 s. Filters with attached structures were deposited in a well plate and submerged in 100% methanol for 10 min, then resubmerged in fresh methanol prior to storage at –20°C until use. The structures were rehydrated with a descending methanol series of 75%, 50%, and 25% methanol in PBS for 5 min each followed by two 10 min equilibrations in PBS and fixed with 2.5% glutaraldehyde overnight at 4°C. Proteinase K treatment was increased to 30 µg/ml for 1 h followed by post-fixation in 4% paraformaldehyde/0.5% glutaraldehyde for 15–20 min. Hybridization was as described (Follstaedt et al., 2003) using 25–50 ng/ml of riboprobe. The number of subsequent washes was increased to 4×15 min in $3 \times$ SSC and 4×15 min in $0.3 \times$ SSC. Anti-DIG was preabsorbed with an acetone powder from *Dictyostelium* cells for 3–4 h at a 1/100 dilution in antibody buffer (PBS, pH 7.4/5% sheep serum/2% BSA). The structures were blocked in antibody buffer for 1 h prior to incubation with preabsorbed anti-DIG at a final concentration of 1/4000 to 1/7000 overnight at 4°C and washed 6×15 min in PBSt-DMSO (PBS, pH 7.4/0.5% Tween-20/2% DMSO). The color reaction with NBT/BCIP was performed from 1–4 h at RT or overnight at 4°C.

Histochemical staining

Plasmids for the prestalk- and prespore-specific promoters *ecmA*, *ecmO*, *ecmA*O, *ecmB*, and *pspA* were kindly supplied by K. Jermyn and J. Williams

and were transformed into the Ax4 or *amtC* null strains by either electroporation (Zinda and Singleton, 1998) or calcium phosphate (De Lozanne and Spudich, 1987; Knecht et al., 1986). Mixing experiments were done between the *amtC* null strain and 10 or 25% wild-type cells obtained from the *Dictyostelium* Stock Center (<http://dictybase.org/StockCenter/StockCenter.html>) that constitutively express β-galactosidase with either the *actin-6* [Ax3K-A6/*lacZ* (HR30)] or the *actin-15* [Ax4-A15/*lacZ* (TL35)] promoters. Additional mixing experiments were conducted between the *amtC* null strain and Ax4 expressing β-galactosidase with the *ecmA* or *ecmB* promoter. Staining for β-galactosidase was carried out as described (Dingermann et al., 1989) with modifications (Richardson et al., 1994). Color substrates were either Bluo-Gal (Sigma) or Magenta-Gal (LabScientific).

Monoclonal anti-STATa (D4; Araki et al., 1998) and purified anti-CudaA (mAB11 (Fukuzawa et al., 1997) 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 plating, harvesting, and rehydrating as described for in situ hybridization. Structures were blocked for 1 h in antibody buffer (PBS, pH 7.4/10% goat serum/2% BSA) before incubation with 1/40× anti-STATa or 1/1200× anti-CudaA overnight at 4°C, and subsequently washed 6×15 min in PBSt-DMSO. Incubation with the secondary antibody, Alexa Fluor 568 (Molecular Probes), was at 1/1800× overnight at 4°C, followed by six further washes with PBSt-DMSO prior to photographing.

Microscopy and image processing

In situ hybridization and β-galactosidase results were photographed with either a Leica MZ16 stereomicroscope with a Q-Imaging Retiga 1300 camera and Q-Imaging or Simple PCI software or with an Olympus AX70 compound microscope with a Q-Imaging Retiga EXi camera and OpenLab software. Fluorescent immunohistochemical results were photographed on the same Olympus microscope and on a Zeiss LSM510 inverted confocal microscope with Zeiss LSM software. Confocal projections of Z-stacks and reslicing to produce new stacks were done with ImageJ (NIH). False color negatives and figures were produced with PhotoStudio (ArcSoft).

Results

Spatial expression pattern of *amtC*

Our initial report on the expression pattern of *amtC* mRNA in developing Ax4 cells as determined by in situ hybridization revealed expression in the prespore region and the tip of the prestalk A region, with the latter only during the first finger and early slug stages (Follstaedt et al., 2003). Using riboprobes, subsequent analysis has indicated that *amtC* has a transient point of increased expression in tight mounds where the tip will form (not shown) that we failed to detect with the less sensitive DNA probes used earlier. Contrary to our initial report, expression was exclusively in the prespore region during the tipped mound and first finger stages and was not seen in the tip until after slug formation, corresponding in timing with genes specific to culmination (Fig. 1). In contrast to our previous claim of loss of tip expression, strong *amtC* expression in tip cells was maintained throughout all stages of culmination (Figs. 1a–d), and weak expression was present in the remainder of the prestalk region. Additionally, *amtC* was expressed in the elongating stalk tube throughout fruiting body formation as can be seen best in Fig. 1b, but expression in mature, vacuolated stalk cells was inconsistent. For mid and late culminants, additional expression was seen in the upper regions of the basal disc cells (d). Prespore/spore expression was as earlier reported. Previously, we demonstrated that when

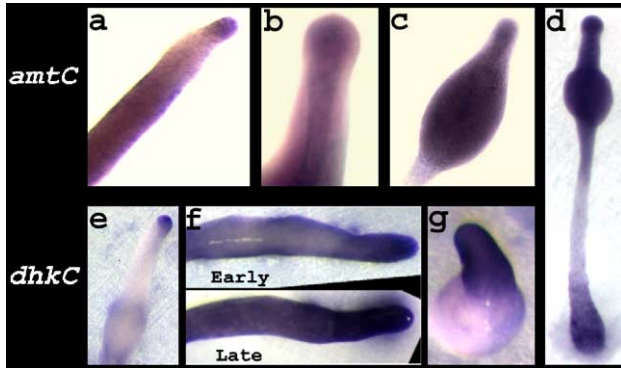


Fig. 1. Spatial localization of *amtC* and *dhkC* mRNAs during development. In situ hybridization was performed with various stages of developing Ax4 cells. Probes specific for either the *amtC* gene or the *dhkC* gene were employed. (a–d) *amtC*; (e–g) *dhkC*; (a, f) slugs; (b) stained stalk tube in early culminant; (c) late culminant; (d) stained basal disk in late culminant; (e) first finger; (g) early culminant. [(a–c) compound microscope; (d–g) stereomicroscope].

starved and developed, cells lacking the *amtC* gene exhibit a slugger phenotype (Follstaedt et al., 2003), and we attributed this defect to the loss of *amtC* expression in the tip cells. Our new in situ results confirming tip expression of *amtC* more closely aligned in timing with stalk tube initiation and culmination strengthen that conclusion.

Prestalk gene expression is delayed in developing amtC null cells

Promoters specific to the prestalk A and O (*ecmA*O), the prestalk A (*ecmA*), the prestalk O (*ecmO*), the prestalk AB (*ecmB*), and the prespore (*pspA*) regions were used to express the β -galactosidase reporter gene to compare cell type-specific gene expression in the *amtC* null strain with that in wild-type. For developing *amtC* null cells, expression of the complete *ecmA* (*ecmA*O) promoter in prestalk A and prestalk O cells was essentially absent at the mound stage (Fig. 2), was expressed at low levels during first finger formation, and remained under-expressed until slugs had migrated for several hours. In contrast, *ecmA*O stained cells in Ax4 were clearly visible in loose mounds, the typical strongly stained prestalk region developed as tips were forming, and this staining pattern was maintained throughout the transitional period and early culmination. Staining in *amtC*[−] structures appeared to initiate behind the tip and on the surface of structures with many cells embedded within the slime sheath during the slug stage (19–24 h). Expression only peaked after 3–6 h of slug migration and increasing numbers of cells were seen in the slime sheath and trail in addition to anterior like cells (ALCs) in the prespore region (24 h). As slug migration time approached 6–10 h, most cells initially expressing *ecmA*O had either ceased expression or were sloughed off into the slime trail, with only mottled staining and a reduced number of ALCs remaining visible (26 h). The prestalk A-specific *ecmA* promoter expression pattern was similar to that of the *ecmA*O promoter (not shown).

Expression with the prestalk O-specific promoter (*ecmO*) revealed that after slugs formed, prestalk O cells were no longer confined to the rear 50% of the prestalk region as is

normal, but were distributed throughout the entire prestalk region (not shown), with increasing frequency as slug migration continued. The expression levels of *ecmO* fluctuated in the same manner as that seen with the *ecmA*O and *ecmA* promoters. Thus prestalk gene expression was delayed, spatially misregulated, and unsustainable in *amtC* null structures. The loss of prestalk gene expression occurred much more rapidly than can be accounted for by the normal loss of prestalk cells and their replacement by prespore cells during prolonged (>30 h) slug migration (Detterbeck et al., 1994; Harwood et al., 1991).

As might be expected, *ecmB* expression was essentially absent in the *amtC* null strain. The majority of slugs had no staining, regardless of the length of time of migration, but

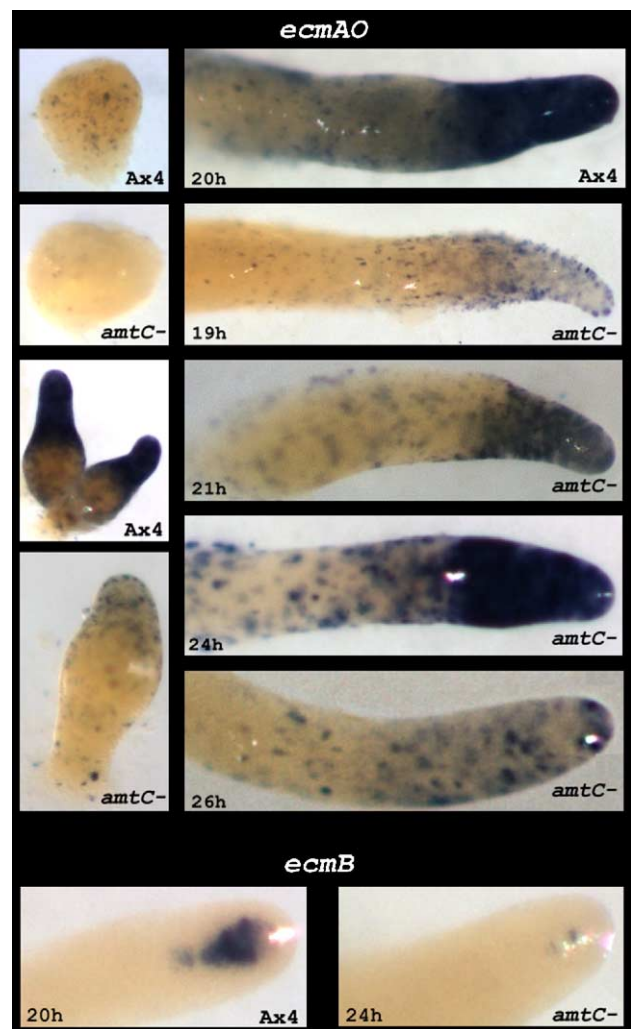


Fig. 2. Prestalk-specific gene expression in developing *amtC* null cells. Wild-type structures are provided for comparison. Ax4 and *amtC* null cells were transformed with constructs possessing the β -galactosidase gene driven by prestalk-specific promoters. Cells were plated for development and structures were harvested and stained for β -galactosidase activity with Bluo-Gal. Promoters and strains are labeled with post-starvation times given for slugs. (*ecmA*O) Mounds (top left), first fingers (middle left), and slugs (top right) showing delayed expression and loss of expression over time; (*ecmB*) slug tips (bottom) showing a typical prestalk AB cone indicating initiation of culmination in wild-type and minimal expression in *amtC*[−]. There was no staining in 85–90% of the *amtC* null slugs.

approximately 10–15% of the slugs had a few cells stained in the appropriate (prestalk AB) region (Fig. 2). RT-PCR analysis confirmed very low to non-existent levels of *ecmB* mRNA in *amtC* null slugs (not shown).

In contrast to the defects seen for prestalk gene expression, prespore gene expression was essentially normal as judged by visualizing *pspA* promoter activity (not shown).

Mixing with wild-type cells does not reliably rescue the slugger phenotype

To determine whether the presence of wild-type cells provided factors that could rescue the *amtC*⁻ slugger phenotype, mixing experiments were conducted with *amtC* null cells and two wild-type strains (Ax4 and KAx3) that were marked by constitutive expression of β -galactosidase with actin promoters. When mixed with 75–90% *amtC* null cells, the vast majority of wild-type cells remained in the prespore region of the structures (Figs. 3a–b), and slug migration usually continued indefinitely as it does when *amtC*⁻ is developed as a monoculture. After migrating for several hours, a few slugs had a concentration of wild-type cells gathered at the tip in the prestalk A* and/or prestalk AB region, and the characteristic transition from a rounded to pointed tip morphology that occurs in Ax4 at the onset of culmination was observed (Fig. 3c). To examine the fate of the Ax4 cells that became established in the prestalk region, *amtC* null cells were mixed with 10 or 25% Ax4-*ecmA::lacZ* or Ax4-*ecmB::lacZ*. In the few slugs with Ax4 cells found in the prestalk region, most of the Ax4 cells became prestalk AB cells, and we believe that these instances accounted for the 5–10% of the structures that culminated in the mixing experiments (not shown).

Cuda is misregulated in the *amtC* null strain

Cuda has a spatial expression pattern (Fukuzawa et al., 1997; Fukuzawa and Williams, 2000) similar to that of *amtC*, with both genes being expressed in the prespore region and the tip of the prestalk A region during the transitional period. Because this expression pattern is rare and *cuda* null strains also have a slugger phenotype (Fukuzawa et al., 1997), we examined *cuda* mRNA expression in developing *amtC* null cells. Expression of *cuda* mRNA in the parental Ax4 strain was constant from the tipped mound stage through culmination



Fig. 3. Localization of wild-type cells when mixed with *amtC* null cells and co-developed. Marked (β -galactosidase) Ax4 or KAx3 cells (10 or 25%) were mixed with *amtC* null cells and plated for development (KAx3 shown). Staining for β -galactosidase activity revealed the position of the wild-type (WT) cells. (a) Tipped mound with WT cells (25%) only in the prespore region; (b) slug with WT cells (25%) predominately localized to the prespore region; (c) late slug with the tipped morphology characteristic of culmination and WT cells (10%) in the prestalk AB and prespore regions.

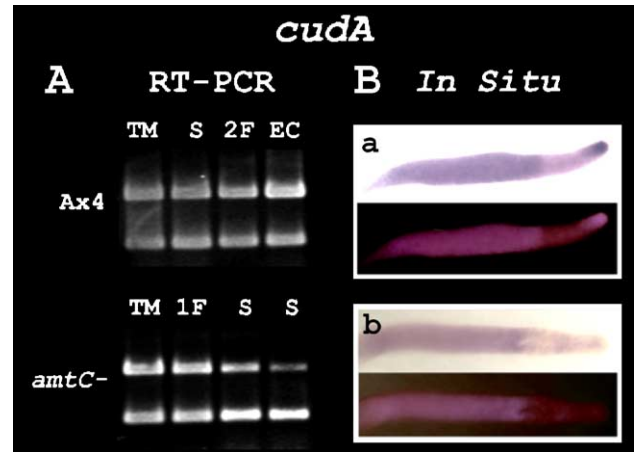


Fig. 4. Expression of the *cuda* gene in Ax4 and the *amtC* null strains. Growing cells of each strain were harvested and plated for development under standard conditions. Structures were collected after various times of development, and either RNA was isolated for RT-PCR analysis (A) or the multicellular structures were fixed and processed for in situ hybridization (B). The upper band in each of the RT-PCR panels corresponds to the *cuda* mRNA while the lower band corresponds to *H7* mRNA as an internal standard (*H7* is expressed at a constant level in growth and at all stages of development). The time points taken for the RT-PCR analysis are: TM, tipped mounds; 1F, first fingers; S, slugs; 2F, second fingers; EC, early culminants. For *amtC*⁻, the first and second slug time points are for just formed slugs and slugs 6 h later. For the in situ panels: (a) Ax4 slug; (b) *amtC*⁻ slug. Slug photographs are the originals with a false color negative beneath it to emphasize the presence or absence of tip expression.

(Fig. 4A). In *amtC*⁻, mRNA levels dropped when slugs were formed, and expression continued to decline as the slugs migrated. This was confirmed by in situ hybridization, with only minimal staining observed after 2–3 h of slug migration (not shown). Significantly, the expression of *cuda* in the tip cells of Ax4 (Fig. 4B(a)) was absent in the *amtC* null strain (b).

To explore Cuda protein distribution, we used immunohistochemical staining. Observed with a compound microscope, *amtC*⁻ appeared to have a complete lack of Cuda protein in the prestalk region (Fig. 5b) in contrast to the strong presence of Cuda in the tip cells of the Ax4 parental strain (a). Consistent with the in situ hybridization results, Cuda was found in near normal levels within the prespore region of early *amtC* null slugs, with the levels declining with increased time of slug migration (not shown).

In addition, we examined Cuda expression using confocal microscopy to determine if low-level expression was present. For Ax4, the presence of Cuda was clearly visible in the prespore cells, prestalk A* cells and AB cone (Fig. 6A). That the stained cells were centrally located at the most anterior tip and in a central core with flanking prestalk cells not expressing Cuda can be seen in the 25 μ m cross-sections in column B and panel E. In the *amtC* null strain, a low level of Cuda protein with a few scattered cells expressing near-wild-type levels was detected throughout the entire prestalk region with no evidence of a central core as was seen in Ax4 (B and E). Thus, in situ hybridization and immunohistochemical staining demonstrated profoundly reduced levels of mRNA and Cuda protein within the prestalk A*/AB cells and low-level ectopic expression throughout the entire prestalk region when *amtC* is absent.

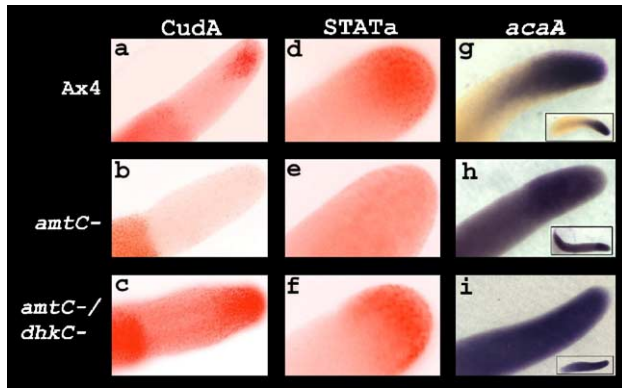


Fig. 5. Spatial localization of the CudA protein (a–c), the STATA protein (d–f), and *acaA* mRNA (g–i) in the prestalk region in the parental and mutant stains as labeled. Growing cells for the indicated strains were harvested and plated for development as described in Materials and methods. Slugs were harvested and fixed after 1–3 h of migration, and immunohistochemical staining or in situ hybridization was carried out. CudA and STATA immunohistochemical staining photographs had similar exposure settings, with exposures for Ax4 and *amtC*⁻ being identical for each antibody. Photographs for CudA and STATA are false color negatives to clarify regions of expression. Punctate staining for CudA and STATA is due to nuclear localization. Non-punctate staining for STATA represents protein within the cytosol. CudA and *acaA* photographs include a portion of the prespore region for reference. Insets of the complete slug are provided for *acaA* photographs. [(a–f) compound fluorescent microscope; (g–i) stereomicroscope].

STATA nuclear localization is absent in the *amtC* null slugs

Because nuclear localized STATA protein induces *cudA* expression in prestalk A*/AB cells (Araki et al., 1998; Fukuzawa and Williams, 2000; Verkerke-van Wijk et al., 2001), the same experiments were carried out with an antibody

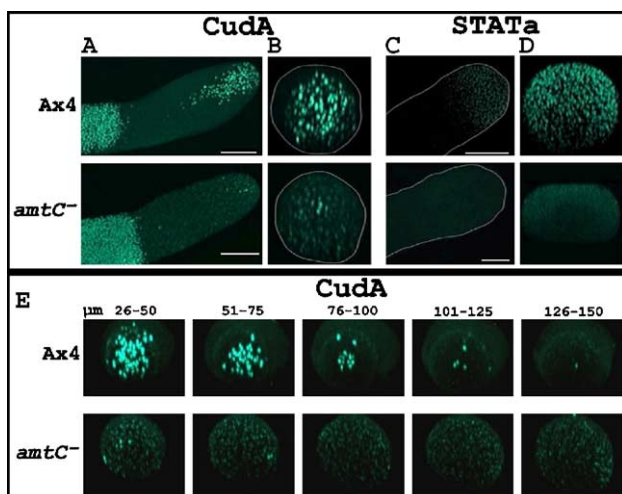


Fig. 6. Confocal analysis of the localization of the CudA and STATA proteins. Confocal settings were identical for each antibody to compare relative intensities. Photographs are pseudo-colored green. (A, C) Projections of original Z-stacks. Scale bars are 50 μm; (B, D, E) ImageJ (NIH) was used to produce a stack of 1 μm slices of the first 150 μm of the prestalk region on a 90° transverse cross-section. Projections were made of successive 25 μm sections. (B, D) The first 25 μm of the tip. (E) Successive prestalk regions projected (26–150 μm) are labeled. Punctate staining for CudA is due to its nuclear localization. Punctate staining for STATA reveals nuclear localized STATA, while non-punctate staining represents STATA within the cytosol.

to STATA. STATA nuclear localization (punctate staining) within the tip of wild-type (Ax4) slugs is shown in Figs. 5d and 6C–D. In *amtC*⁻, variable levels of diffuse accumulation of STATA in the tip were seen (Figs. 5e and 6C–D), but at no point did it become nuclear localized. These results suggest that the lack of CudA in the tip cells of *amtC* null slugs results from a lack of nuclear localized STATA protein. Using RT-PCR, we confirmed that *STATA* mRNA was being produced in normal amounts in the *amtC* null strain (not shown).

Other STATA induced genes are underexpressed

As the above results indicated that STATA nuclear localization did not occur in the *amtC* null slugs, we examined other genes normally expressed in the prestalk region that are induced by STATA. Recent work showed that *SLF308*, encoding an extracellular matrix protein, is expressed under the control of nuclear localized STATA in prestalk A cells from first fingers onward (Shimada et al., 2004). RT-PCR for *SLF308* indicated that little to no expression occurred in the *amtC* null strain (Fig. 7). *asIA*, a homologue to Acetyl-CoA synthetase, is induced by STATA in the prestalk AB cone from the first finger through early culminant stages and is repressed in prestalk A and O cells in the mound and first finger stages by a repressor known not to be STATA (Shimada et al., 2005). When examined by RT-PCR in *amtC* null cells during the mound, first finger, and slug stages, there was a reduced level of *asIA* mRNA during the first finger stage and expression was essentially absent in slugs (Fig. 7).

Expression of *acaA* is altered in the *amtC* null strain

From in situ analysis, it has been inferred that ACA localized to the tips of wild-type slugs generates the extracellular cAMP necessary for the nuclear localization of STATA during the transitional period (Araki et al., 1998; Dormann et al., 2001b; Fukuzawa and Williams, 2000; Verkerke-van Wijk et al., 2001). Therefore, we examined *acaA* mRNA distribution using in situ hybridization. During slug migration, expression in Ax4 cells decreased as expected in the prespore region and became tip-specific by the late slug stage (Fig. 5g). In *amtC* null slugs, *acaA* mRNA levels and localization varied with

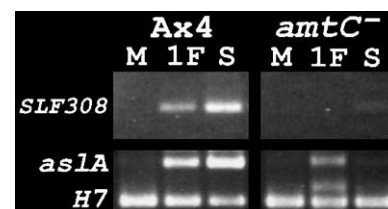


Fig. 7. RT-PCR analysis in Ax4 and the *amtC* null strain for STATA dependent genes. Growing cells of each strain were harvested and plated for development under standard conditions. Cells were collected at various times after plating followed by RNA isolation. M, tipped mounds; 1F, first fingers; S, slugs. (*SLF308*) *H7* could not be used because of overlap in band size; (*asIA*) *H7* mRNA was used as an internal control. The faint middle band in the *amtC*⁻ 1F lane is of unknown origin but was seen sporadically in both Ax4 and *amtC*⁻ upon repeat experiments.

frequent overexpression seen in early slugs (h). As slugs migrated, most eventually lost all expression. Significantly, overexpression of *acaA* mRNA in the *amtC* null slugs was at a time when *CudA* expression and STATA nuclear localization were absent in the prestalk region, suggesting that cAMP should have been sufficient for STATA to translocate to the nucleus to induce *cudA* expression.

Ectopic CudA expression restores culmination

CudA in prestalk A* cells is required for culmination (Fukuzawa et al., 1997), and we find it is underexpressed in the *amtC* null slugs and essentially absent in anterior tip cells, presumably due to the lack of STATA nuclear localization. We transformed both strains of *amtC*⁻ with constructs expressing the *cudA* gene with different spatially expressed promoters to examine possible rescue of the slugger phenotype. These were the ubiquitously expressed *actin-15* promoter, the prestalk-specific *ecmA* promoter, and the prespore-specific *pspA* promoter. RT-PCR was used to check the expression levels of *cudA* mRNA in the transformed strains at the slug stage (Fig. 8A). Minimal expression of *cudA* was seen using the *actin-15* and the *ecmA* promoters, while the *pspA* promoter resulted in high levels of *cudA* mRNA, but presumably only in prespore cells. When the strains were developed, *CudA* expression driven by the *pspA* promoter provided no rescue (Fig. 8B), and slugs migrated indefinitely. In contrast, the *actin-15* and *ecmA* promoters (Fig. 8B) substantially restored culmination, although the transitional period tended to be longer than that for wild-type cells. Strains expressing *CudA* with the *ecmA* promoter had a somewhat longer second finger/early culminant stage than those with the *actin-15* promoter, and culmination was asynchronous. Thus, expression of *CudA* in the appropriate cell types (i.e., the anterior cells) partially restored culmination in the *amtC* null strain in the absence of nuclear localized STATA.

Loss of DhkC rescues the slugger phenotype of the amtC null strain

The slugger phenotype of the *amtC* null strain suggested to us that AmtC might function by mediating control of the DhkC phosphorelay by ammonia. To initiate culmination in response to the appropriate environment, AmtC might be necessary to inhibit the DhkC phosphorelay by either generating locally low ammonia levels, sensing when levels are low, or both. Thus, in the *amtC* null slugs, the phosphorelay would remain inappropriately active and the cAMP phosphodiesterase activity of RegA would prevent activation of PKA, and hence initiation of culmination. If true, a strain disrupted in both *amtC* and *regA* should result in the *regA*⁻ phenotype of precocious sporulation (Shaulsky et al., 1996; Shaulsky et al., 1998; Thomason et al., 1998). Indeed, disruption of *amtC* in a *regA* null background resulted in a premature sporulation phenotype that was indistinguishable from the parental *regA*⁻ strain (not shown).

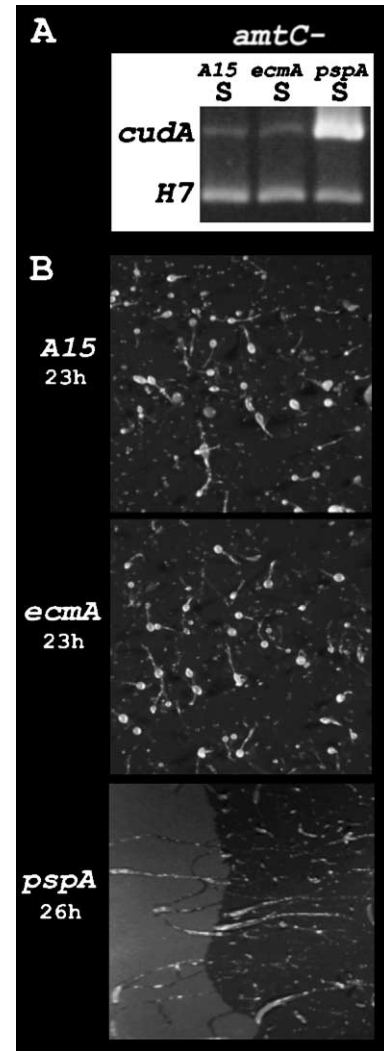


Fig. 8. Morphology of the developing cells of the *amtC* null strain transformed with *cudA* expressing plasmids. The *amtC* null strain was transformed with plasmids that expressed *cudA* using either the *actin-15* promoter (ubiquitously expressed to varying extents in all cell types), the *ecmA* promoter (expressed in prestalk A cells), or the *pspA* promoter (expressed in prespore cells). Growing cells of each strain were harvested and plated for development under standard conditions. (A) RNA was harvested from cells at the early slug stage (15–16 h post-starvation) and RT-PCR analysis was performed to examine the expression of the *cudA* mRNA. *H7* was used as an internal control; (B) photographs were taken at the indicated times post-starvation and show varying stages of culmination for the *actin-15::cudA* and *ecmA::cudA* cells, while the *pspA::cudA* cells remain migrating slugs.

As RegA also plays important roles in cAMP metabolism at earlier stages that are unrelated to the DhkC phosphorelay (Laub and Loomis, 1998; Maeda et al., 2004), we further investigated a potential relationship between AmtC and DhkC by disrupting the *dhkC* gene in the *amtC* null background. In the *amtC*⁻/*dhkC*⁻ strain, culmination was restored for the majority of structures after a short period of slug migration (Fig. 9). The transitional slug stage in the *amtC/dhkC* double null that is absent in the *dhkC* null strain (Singleton et al., 1998) is likely due to effects of loss of AmtC activity that may not be mediated or additionally affected by the DhkC phosphorelay. Nonetheless, the rescue

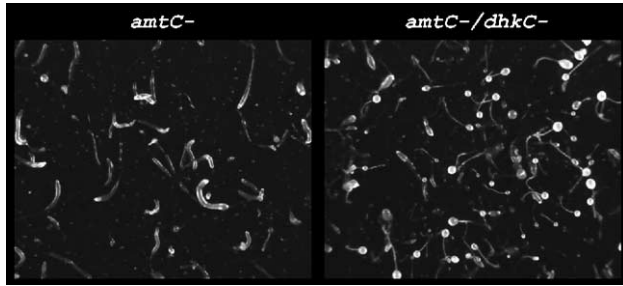


Fig. 9. Morphology of developing cells of the *amtC* null and *amtC/dhkC* double null strains. Growing cells were plated under standard conditions of development and photographed 23 h post-starvation at 1.25 \times . Numerous culminating structures were present for the *amtC*⁻/*dhkC*⁻ strain while *amtC*⁻ cells remained migrating slugs.

of the slugger phenotype indicates that AmtC is an upstream regulator of the DhkC phosphorelay during the transitional period.

Expression of dhkC is upregulated in the tip during the transition from slug to culminant

Because the role of DhkC in regulating the slug/culmination choice predicts that expression of *dhkC* should occur within tip cells, we examined the spatial expression of *dhkC* in Ax4 cells by in situ hybridization (Fig. 1). Mounds displayed weak expression of *dhkC* within the upper regions and very strong expression at the perimeter of basal cells in contact with the substrate (not shown). Expression became tip-specific during first finger formation with low expression levels seen in the posterior portion of the prespore region (Fig. 1e). During slug migration, prespore expression levels increased moderately and expanded anteriorly to the entire prespore region, while the more strongly stained tip region enlarged to the entire prestalk zone (f). Expression levels increased with further migration. As second fingers and early culminants were formed, *dhkC* expression was lost from the prespore region in an anterior to posterior progression, while strong expression in all prestalk cells was maintained (g).

Loss of the dhkC phosphorelay in amtC null cells restores normal STATA nuclear localization

AcaA mRNA expression, STATA nuclear localization, and CudA protein expression were examined in the *amtC/dhkC* double null strain. Unexpectedly, *acaA* mRNA was over-expressed throughout the entire double null slug (Fig. 5i), suggesting the presence of elevated ACA levels and cAMP production in all regions of the slug. Localization of STATA within the nucleus in the *amtC/dhkC* double null strain was restored and limited to the tip (f) as is seen in wild-type, despite ectopic expression of *acaA*. CudA was present in high levels in an enlarged region that encompassed the normally smaller region of localization composed of prestalk A* cells and the prestalk AB cone (c). In addition, a moderate level of CudA also was found throughout the remainder of the prestalk region. In situ hybridization confirmed that *cudA* expression was

present throughout the entire slug in the *amtC*⁻/*dhkC*⁻ strain (not shown).

Discussion

The slugger phenotype of the *amtC* null strain was rescued by inhibiting the DhkC phosphorelay through disruption of either the *regA* or the *dhkC* gene. This finding supports the hypothesis that AmtC normally inhibits the DhkC phosphorelay when conditions are conducive for culmination, i.e., low local ammonia levels (Fig. 10). The lack of tip-specific nuclear localization of STATA in *amtC* null slugs and its restoration in the double null strain further supports this hypothesis, and confirms that loss of AmtC function does not intrinsically prevent translocation of STATA to the nucleus. In the *amtC* null slugs, even under environmental conditions that are conducive for culmination, DhkC would remain inappropriately active due to an inability to sense low local ammonia levels in the absence of AmtC and/or due to ammonia levels becoming increasingly high as a result of no AmtC transporter activity. In

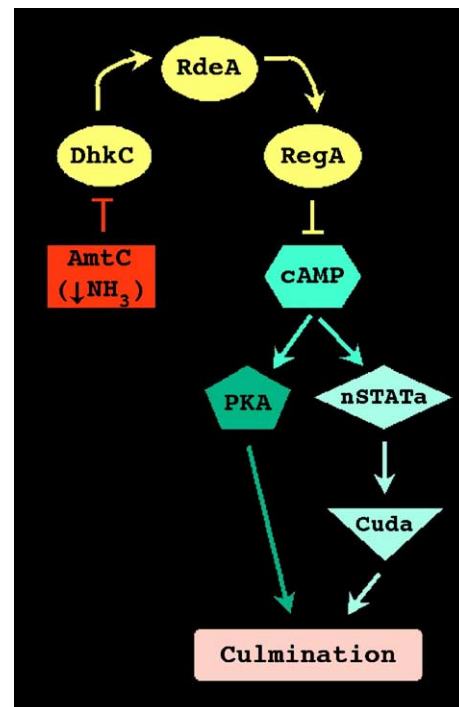


Fig. 10. A model of the regulation of the slug/culmination choice by AmtC. When active, DhkC phosphorylates itself and passes the phosphate through the relay to RegA, activating RegA's cAMP phosphodiesterase activity. The result is low cAMP and slug migration instead of culmination (Singleton et al., 1998). High cAMP is required for culmination: intracellularly to activate PKA and extracellularly to cause nuclear localization of STATA and subsequent production of CudA. The results presented herein lead to the proposal that AmtC normally inhibits the DhkC phosphorelay when conditions are conducive for culmination, that is, when local ammonia levels are low. Alternatively, AmtC may generate the low local ammonia levels, via export from tip cells, which are needed to inhibit the phosphorelay. Whether AmtC functions by one or both of these mechanisms remains to be determined, but in either case, culmination is blocked in the *amtC* null strain due to the lack of proper inhibition of the DhkC phosphorelay. Artificially removing the phosphorelay by disruption of *dhkC* or *regA* restores culmination.

either case, as indicated in the model (Fig. 10), the result would be an overactive DhkC phosphorelay, resulting in RegA hydrolysis of intracellular cAMP faster than it can be exported to initiate the STATA nuclear localization process. Additionally, the reduced concentration of intracellular cAMP would result in continual suppression of PKA activity, and this suppression combined with the lack of nuclear STATA and *cudA* induction in tip cells would contribute to an inability to culminate (Mohanty et al., 1999; Singleton et al., 1998). These defects would be overcome by removal of either RegA or its upstream activator, DhkC, and indeed disruption of the *dhkC* gene in the *amtC* null strain restored nuclear localization of STATA, CudA expression, and culmination.

In the double null strain, nuclear localization of STATA was restored in the tip with an identical spatial pattern as that of wild type, despite ectopic and overexpression of *acaA* mRNA. Although mRNA levels do not always reflect protein expression or activity levels, if excess cAMP is being produced throughout the double null slugs as these results suggest, the restriction of STATA nuclear localization to the tip cells would indicate that a secondary control mechanism exists that is independent of AmtC and DhkC that either limits the export of cAMP to the tip or limits ACA activity to the tip, irrespective of *acaA* expression. By one or the other mechanism, sufficiently high extracellular cAMP concentrations necessary for STATA translocation to the nucleus (Dormann et al., 2001a) occurred only in the tips of the double null slugs.

The over and ectopically expressed *acaA* mRNA in the double null slugs contrasts with the loss of expression in *amtC*⁻ slugs after long periods of migration. In some instances, *amtC*⁻ slugs lost *acaA* expression in the prestalk A*/AB region prior to losing it in the remainder of the slug. These findings suggest that AmtC, and perhaps DhkC, may play a role in regulating *acaA* expression during the transitional period. Earlier studies demonstrated an effect of ammonia or ammonia signaling on intracellular and extracellular cAMP production (Riley and Barclay, 1990; Schindler and Sussman, 1979; Sussman et al., 1978; Williams et al., 1984), and our previous (Singleton et al., 1998) and current results suggest that this may be due, in part, to AmtC mediation of PKA activity via the DhkC phosphorelay and subsequent effects on *acaA* expression.

CudA expression in the *amtC/dhkC* null strain also distinctly differed from that in the parental Ax4 and *amtC* null strains. High levels of CudA were restored in the entire tip where STATA nuclear localization had been restored, and in an enlarged and no longer conical shaped region where the prestalk AB cells are normally located. This represents an expanded region of high CudA expression because normally in Ax4, prestalk expression is confined to the prestalk A* cells and the AB cone. The results indicate that CudA expression in the double null is a response to the restoration of STATA nuclear localization in the tip. Additionally, the ectopic expression in the *amtC* and *amtC/dhkC* null strains suggests a possible defective repression mechanism that may normally limit CudA expression to prestalk A*/AB cells instead of all prestalk A cells. Studies on the promoter structure of the *cudA*

gene reveal a general activator that has the potential to direct *cudA* expression in all cells of the slug (Fukuzawa and Williams, 2000). In addition, there is a prestalk repressor that normally prevents expression in prestalk cells but that does not function in prestalk A*/AB cells, presumably due to STATA (Fukuzawa and Williams, 2000; Verkerke-van Wijk et al., 2001). The prestalk repressor may not be fully functional in the absence of AmtC, resulting in the observed low ectopic expression of CudA throughout the entire prestalk region of *amtC* null slugs. Why there was an increase in the level of ectopic CudA expression with the additional loss of the DhkC phosphorelay is puzzling, but suggests other influences on the repressor by cAMP or PKA activity.

Our investigation of the influence of AmtC on the slug/culmination decision during the transitional period was complicated by defects in prestalk differentiation and gene expression in the *amtC*⁻ strain. Although initial cell type proportioning appeared normal in developing *amtC* null cells when examined with the prespore *pspA* promoter, prestalk gene expression as evidenced by the paradigmatic *ecmA*O promoter was both delayed and not maintained after “normal” expression levels had been reached. In addition, *ecmO* was expressed in the prestalk A region soon after slugs began migration. In tight mounds, *amtC* mRNA was expressed in all wild-type cells with a point of increased expression where the tip forms and was spatially discrete thereafter. This suggests that the delayed and spatially inappropriate expression of *ecmA*O was a direct result of the loss of AmtC function.

Additionally, the absence of *ecmB* expression in the *amtC* null strain diverges from that seen in both wild-type and *STATA* null cells. In the *STATA* null strain, *ecmB* is expressed ectopically in prestalk A cells due to the lack of STATA inhibition (Mohanty et al., 1999). Because STATA is not present in the nucleus of prestalk A cells in *amtC* null slugs, ectopic expression of *ecmB* in the prestalk A region of this strain would be expected. Instead, we found essentially no expression of *ecmB*, either ectopically or at levels normally seen in prestalk AB cells in the wild-type strain when culmination is initiated. Previous work has demonstrated that ammonia can repress *ecmB* expression (Wang et al., 1990), and our findings suggest that AmtC may mediate this effect of ammonia. Studies of the *ecmB* promoter reveal that an uncharacterized transcriptional activator is needed for *ecmB* expression in the prestalk AB core (Ceccarelli et al., 1991; Harwood et al., 1993), and AmtC may be necessary for the proper function of this activator. Similar to the situation in the *amtC* null strain, *ecmB* is not expressed in slugs formed by cells possessing a constitutively active DhkC protein (Singleton et al., 1998). This suggests that an inappropriately active DhkC phosphorelay is preventing *ecmB* expression in the *amtC* null slugs, perhaps due to the resultant suppression of PKA activity. Further experimentation is required to distinguish among these possibilities and to determine the nature of the requirement of AmtC for normal *ecmB* and *ecmA*O expression.

In sum, our findings demonstrate a role for the ammonium transporter AmtC in regulating the slug/culmination choice and in influencing prestalk cell differentiation and gene expression.

The aberrations in the *amtC* null strain, particularly the lack of nuclear localization of STATa and *CudA* expression, appear to be related to previously demonstrated PKA or cAMP dependent events, suggesting that AmtC regulates these events via the DhkC phosphorelay (Fig. 10). We have argued that the aberrations in the *amtC* null strain result primarily from AmtC not being present to inhibit the DhkC phosphorelay when conditions are conducive for culmination, that is, when local ammonia levels are low. In the *amtC* null strain, the slugger phenotype indicates that the anterior cells sense either a real or perceived continual high local concentration of ammonia, thus locking them into the slug stage. The question remains as to whether the ammonia concentration increases in the absence of AmtC or whether there is a false perception of high ammonia concentrations resulting in the overstimulation of the DhkC phosphorelay. In other words, does AmtC function to remove ammonia at the appropriate time from tip cells and hence release ammonia's inhibitory effect on the initiation of culmination (Schindler and Sussman, 1977; Schindler, 1978; Singleton et al., 1998)? Or alternatively (or in addition), does AmtC function to sense the external ammonia concentration and, in response to low concentrations, initiate culmination by inhibition of the DhkC phosphorelay?

In yeast and pathogenic fungi, ammonium transporters have been shown to function as ammonia sensors in signal transduction processes that mediate ammonia's role as a regulator of physiological events (Lorenz and Heitman, 1998; Smith et al., 2003). Ammonium transporters also participate in signal transduction pathways in many bacteria where their activity can be modulated via such pathways in response to ammonia (or nitrogen) levels (Coutts et al., 2002). Recent diffraction studies on crystals of the ammonium transporter AmtB of *E. coli* demonstrate that ammonium transporters possess an extracellular binding site for an ammonium ion (Khademi et al., 2004; Zheng et al., 2004). After binding, a proton is removed and the "transporter" acts as a channel to pass the ammonia, a mechanism that allows both import and export. The amino acids that make up the ammonium binding site are generally conserved in various AMTs, and in particular, they are present in AmtC of *Dictyostelium*. The existence of an extracellularly located ammonium binding site fits well with the possibility of AmtC serving as an ammonia sensor. Mutational analysis based on the crystal structure is in progress to differentiate between AmtC functioning simply as a transporter or additionally as a sensor of ammonia.

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