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The Hybrid Histidine Kinase *dhkB* Regulates Spore Germination in *Dictyostelium discoideum*

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Spore germination is a defined developmental process that marks a critical point in the life cycle of *Dictyostelium discoideum*. Upon germination the environmental conditions must be conducive to cell growth to ensure survival of emerged amoebae. However, the signal transduction pathways controlling the various aspects of spore germination in large part remain to be elucidated. We have used degenerate PCR to identify *dhkB*, a two-component histidine kinase, from *D. discoideum*. *DhkB* is predicted to be a transmembrane hybrid sensor kinase. The *dhkB*-null cells develop with normal timing to give what seem to be mature fruiting bodies by 22 to 24 h. However, over the next several hours, the ellipsoidal and encapsulated spores proceed to swell and germinate *in situ* within the sorus and thus do not respond to the normal inhibitors of germination present within the sorus. The emerged amoebae dehydrate due to the high osmolarity within the sorus, and by 72 h 4% or less of the amoebae remain as spores, while most cells are now nonviable. Precocious germination is suppressed by ectopic activation of or expression of cAMP-dependent protein kinase A. Additionally, at 24 h the intracellular concentration of cAMP of *dhkB*$^-$ spores is 40% that of *dhkB*$^+$ spores. The results indicate that DHKB regulates spore germination, and a functional DHKB sensor kinase is required for the maintenance of spore dormancy. DHKB probably acts by maintaining an active PKA that in turn is inhibitory to germination.

INTRODUCTION

Although best known for the multicellular developmental pathway, *Dictyostelium* cells also undertake several other developmental programs given the appropriate environmental and cellular status. These include a sexual pathway leading to macrocyst formation (Loomis, 1975) and the pathway of spore germination that results in amoebae with an activated program of cell growth and cell division (Cotter *et al.*, 1992). Spores are produced during the multicellular developmental program that results in a stalk-supported sorus packed with dormant spores. The spores, which are resistant to various environmental assaults, typically germinate upon dispersal and under appropriate environmental conditions.

The spore germination program is a critical point in the life cycle of *Dictyostelium* as it results in the reappearance of vegetative amoebae that will grow and propagate. The environmental conditions appropriate for germination must also be conducive to cell growth to ensure survival of emerged amoebae. Spores are held in a dormant state within the sorus of the mature fruiting body to ensure that spores do not germinate until they are dispersed. Spore germination is inhibited by discadenine, produced during spore maturation and localized to the spore matrix, and the high osmotic pressure that develops in the sorus (Abe *et al.*, 1976; Ceccarini and Cohen, 1967; Cotter *et al.*, 1992). Upon dispersal, the discadenine is diluted, and the spores undergo germination in response to nutrients and bacterial factors in the surrounding environment (Cotter, 1981; Hashimoto, 1976; Katilus, 1975). Additionally, after 10–14 days in the sorus *Dictyostelium* spores acquire the ability to autoactivate the germination process upon removal of discadenine even in the absence of any exogenous growth factors (Cotter, 1989; Dahlberg, 1978). This is presumably a survival response since spores begin to lose viability after 14 days in the fruiting body.

Spore germination is a defined developmental process that consists of four distinct stages: activation, postactivation lag, spore swelling, and emergence of amoebae from the spore (Cotter, 1981). As with any developmentally regulated pathway, changes in protein synthesis and mRNA expression correspond with the various stages of the morphogenesis of spores into amoebae (Dowbenko and Ennis, 1980; Giri

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and Ennis, 1977, 1978). The process of spore germination can be deactivated, returning the spores to a dormant state. Deactivation of the germination pathway occurs in response to harsh environmental conditions, such as extreme temperatures and pH, high osmotic pressure as in the sorus, and low oxygen levels, to prevent amoebae from dying upon emergence (Cotter *et al.*, 1979; Glaves and Cotter, 1989). The reversal of activated spores to a dormant state can only occur before the swelling stage as this marks an irreversible step in germination (Cotter and Raper, 1968). The signal transduction pathways controlling the various aspects of spore germination in *Dictyostelium* in large part remain to be elucidated.

Two-component signal transduction systems are used by eukaryotes and prokaryotes to sense various environmental signals and elicit appropriate physiological responses including chemotaxis (Bourret *et al.*, 1991), virulence (Parkinson and Kofoid, 1992), and development (Errington, 1993). The most basic of these systems is composed of two proteins, the sensor or histidine kinase and the response regulator (Alex and Simon, 1994; Parkinson and Kofoid, 1992). The sensor is composed of an input domain and the histidine kinase domain, while the response regulator is made up of a receiver domain and an output domain. The sensor protein perceives the signal from the environment through the input domain, resulting in the autophosphorylation of a conserved histidine residue within the histidine kinase domain. The phosphate is then transferred to a specific aspartate residue of the receiver domain within the response regulator. The phosphorylated response regulator brings about a precise physiological change through the activity of the output domain, often by regulating gene expression. In prokaryotes the output domain is often a DNA binding protein that can directly alter gene expression and cell physiology. In eukaryotes the best-characterized two-component systems feed into more traditional eukaryotic signaling pathways such as a MAP kinase cascade in order to elicit a physiological response (Chang, 1996; Posas *et al.*, 1996).

In *Dictyostelium* several two-component signaling proteins have been identified. The histidine kinase DOKA was found to be involved in sensing osmolarity and is involved in spore maturation (Schuster *et al.*, 1996). The two-component sensor kinase DHKA is necessary for proper spore formation and stalk morphogenesis (Wang *et al.*, 1996). Additionally, the putative response regulator REGA is a negative regulator of spore formation (Shaulsky *et al.*, 1996). Using degenerate oligonucleotides and PCR amplification, we have identified three other histidine kinase genes in *Dictyostelium discoideum*. Here we describe the molecular cloning and characterization of *dhkB*. From amino acid sequence comparisons, *dhkB* is predicted to be a transmembrane sensor kinase. Although *dhkB*-null cells develop with normal timing to give what seem to be mature fruiting bodies by 22 to 24 h, over the next several hours the ellipsoidal and encapsulated spores proceed to swell and germinate *in situ* within the sorus. The emerged amoebae dehydrate due to the high osmolarity within the sorus, and by 72 h 4% or less of the cells remain as spores while most amoebae are now nonviable. Thus, DHKB regulates spore germination, and a functional DHKB sensor kinase is required for the maintenance of spore dormancy, probably by contributing to the maintenance of an active cAMP-dependent protein kinase (PKA) in dormant spores.

MATERIALS AND METHODS

Cloning and sequencing of dhkB. The consensus amino acid sequence of the H and G2 motifs of several histidine kinase domains was determined and used to design degenerate oligonucleotides, taking into account the codon bias of *Dictyostelium*. The two oligonucleotides 3′ 2C (ARNCCNARNCCNGTNCC) and 5′ 2C (TCNCAYGARYTNMGNACNCC) were used to perform RT–PCR (Pekovich *et al.*, 1998) on total RNA isolated from growing KAx3 cells. The resulting PCR products were cloned into pGEM-1 (Promega) and sequenced using the Fidelity DNA sequencing system (Oncor Appligene). A BLAST search on the 560-bp sequence of one clone confirmed sequence identity with histidine kinase domains from other genes. The *dhkB* kinase domain was subsequently used to screen and clone a 6711-bp genomic fragment containing the majority of the *dhkB* ORF. The genomic library used was a generous gift from the laboratory of Dr. R. Firtel.

The remaining 3′ end of *dhkB* was isolated by 3′ RACE (Frohman *et al.*, 1988; Ohara *et al.*, 1989). 3′ RACE was carried according to the Superscript Preamplification System for first-strand cDNA synthesis (Gibco BRL). Briefly, 3′ RACE RT primer was used in a reverse transcriptase reaction with 5 μg of either 0 h poly(A)$^+$ RNA or 0 h total RNA and Superscript II RTase (Gibco BRL) for first-strand cDNA synthesis. This was followed by RNase H treatment and standard PCR on 10% of the first-strand cDNA. The 3′ RACE product was amplified with the C3-R2 (ATGTAGCACACAATGGAGTAGAAGC) and 3′ RACE RT (CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC$(T)_{15}$VN) primers.

The *dhkB* sequence is deposited in GenBank, Accession No. AF024654.

dhkB gene disruption. A 2000-bp *Nde*I/*Bgl*II fragment was released from the *dhkB* genomic clone and replaced with a 1.4-kb Blasticidin S resistance cassette (Sutoh, 1993): pBSR519 was a generous gift from Dr. F. Puta. Exponentially growing KAx3 cells in axenic broth (between 1×10^6 and 4×10^6 cells/ml) were transformed by electroporation as described (Howard and Firtel, 1988) with the linearized disruption construct p2C3/Bsr. The transformations were then selected on 10 or 40 μg/ml Blasticidin S (ICN), changing the medium and Blasticidin S three times over the 10 days of selection. On the tenth day transformants were washed once with HL-5, harvested, and plated down clonally with *K. pneumoniae* on SM plates (Sussman, 1966) and allowed to grow at 21°C. Individual colonies were picked and transferred to SM plates with a *K. pneumoniae* lawn. Similarly, *dhkB*$^+$ and *dhkB*$^-$ cells were transformed with a SP60::PKAcat construct (Mann *et al.*, 1994; a generous gift from R. Firtel), and transformants were selected using G418.

Cell and spore viability assays. Spore viability was determined as described (Hadwiger and Firtel, 1992). Cells were allowed to develop for 29 to 72 h. The terminal structures were resuspended in 1 ml of 10 mM EDTA, pH 7.2, and 0.1% NP-40 and incubated at 42°C for 45 min. The spores were washed 3× with 1 ml of PDF and finally resuspended in 1 ml of *K. pneumonia* culture. The

spores were then diluted and plated on SM plates and incubated at 21°C until colonies began to appear. The colonies were counted and $dhkB^+$ and $dhkB^-$ survivors were compared. Cell viability was determined by picking several sori from fruiting bodies at various points after the initiation of development and then resuspending in PDF. The total number of cells (all types) was determined and cells were plated at various titers with *K. pneumonia* on SM plates. The number of colonies were counted and $dhkB^+$ and $dhkB^-$ survivors were compared.

Osmotic shock experiments. Osmosensitivity was determined as described (Schuster *et al.*, 1996). *Dictyostelium* cells were grown on *K. pneumonia* until clearing became evident. The cells were separated from the bacteria by differential centrifugation and resuspended in PDF at 3.0×10^7 cells/ml. Two milliliters of cells were placed in several tubes and placed on a shaker at 200 rpm and 21°C for 1 h. The cells were spun down and resuspended in either PDF, ddH_2O, or 400 mM sorbitol-PDF, followed by further shaking for 120 min. The cells were plated with *K. pneumonia* at ~50 and 500 cells/plate and incubated at 21°C for 3–4 days and the resulting colonies were counted.

RNA isolation and analysis. Total RNA was isolated from growing cells developed for various times as described (Singleton *et al.*, 1987). Probe generation, Northern analysis, and hybridizations were carried out as described previously (Singleton *et al.*, 1987). RT–PCR analysis of *dhkB* mRNA was performed as described (Pekovich *et al.*, 1998). Briefly, 3′ primers specific for *dhkB* and the H7 gene (Zhang, 1995) were used to make cDNA from total RNA isolated from growing cells and cells plated for development for various times. H7 served as an internal control; it is expressed constitutively during growth and development. The 5′ primers specific to each gene were added along with the appropriate buffer and salts, and amplification was carried out for 18, 20, 22, and 24 cycles. One microcurie of [α-^{32}P]dCTP was included to label the amplified bands. Following separation by gel electrophoresis and drying of the gel, the bands were visualized and quantitated using a Molecular Dynamics Phosphorimager. Quantitation was normalized to the constitutively expressed H7 gene, and the time point producing the strongest normalized *dhkB* signal was set at 100%. All other values are relative to this. The values obtained for those cycle numbers that gave a linear increase (usually 20, 22, and 24) were averaged and were used in generating the figure.

Spore staining. Calcofluor (Sigma) was used to stain for cellulose within the spore coat. Total cells from the *dhkB*-null and wild-type sori were incubated at room temperature for 3 min with a 0.001% w/v solution of calcofluor in PDF. The cells were then washed three times with PDF to remove any background staining. The stained cells were visualized and captured on a Zeiss Axioplan 2 using a 40× Neofluar objective and No. 5 filter for fluorescence. Use of and assistance with the microscope were provided by Dr. D. Schot, Department of Surgery.

cAMP assays and suppression of germination by 8-bromo-cAMP. Intracellular levels of cAMP in spores collected 24 h after plating cells for development were determined with a cAMP RIA kit (Amersham). Spores (1.5×10^7) were washed and were suspended in 3.5% perchloric acid. The suspension was incubated at 37°C for 15 min. Following neutralization, cAMP amounts were determined according to the instructions supplied by the manufacturer. Two to four measurements were made for each experiment, and three independent experiments were performed.

The effect of 8-bromo-cAMP on germination of $dhkB^-$ spores was performed as follows. Spores were collected at 24 h and were suspended at 10^7 spores per milliliter in HL-5 medium with or without 23 mM 8-bromo-cAMP. The suspensions were shaken at room temperature, and the number of spores and emerged amoebae were counted by microscopic examination every 2 h.

RESULTS

Cloning and sequencing of dhkB. The histidine kinase module of the two-component sensor protein contains five amino acid motifs that are highly conserved among all histidine kinase domains (Parkinson and Kofoid, 1992). The H motif contains the histidine moiety that is the site of autophosphorylation. The G1 and G2 motifs are involved in nucleotide binding and the N and F motifs have no known function. Degenerate PCR primers were designed based on the amino acid sequence of the H and G2 motifs (Fig. 2A). These primers were used in a reverse transcriptase–polymerase chain reaction (RT–PCR) with total RNA isolated from *Dictyostelium* amoebae growing axenically.

A fragment of 560 bp was cloned and sequenced and designated *Dictyostelium* histidine kinase B (*dhkB*). The fragment possessed an ORF that contained the remaining three conserved histidine kinase motifs inside the primer regions and that showed significant sequence identity with other eukaryotic and prokaryotic histidine kinase domains. The degenerate PCR fragment was used to generate a probe and screen a genomic library. A 6711-bp genomic clone was identified and sequenced in its entirety. The sequence revealed an ORF that began 1058 bp from the 5′-end and continued to the 3′-end except for a single intron of 80 bp starting 2370 bp into the coding sequence. The exon–intron boundaries were confirmed by RT–PCR on total RNA isolated from growing cells using primers on each side of the intron and sequencing the PCR product directly. The genomic clone did not contain the entire coding sequence, and therefore 3′ RACE (Frohman *et al.*, 1988; Ohara *et al.*, 1989) was employed to isolate and sequence the remainder of the gene (ca. 400 bp). The composite sequence was 7133 bp (data not shown) in length with an ORF corresponding to a 219-kDa protein of 1969 amino acids (Fig. 1).

Predicted amino acid sequence of DHKB. The amino acid sequence of DHKB revealed that it contains an amino-terminal input domain followed by a histidine kinase domain and finally a carboxy-terminal receiver domain (Fig. 1). This arrangement of domains is referred to as a hybrid histidine kinase. DHKB shows clear sequence identity with the histidine kinase (Fig. 2A) and receiver (Fig. 2B) domains from the hybrid histidine kinases identified in *Dictyostelium* and other eukaryotes. Several hybrid kinases are found in bacteria, and all but one of the identified eukaryotic sensors are hybrid kinases. A BLAST search revealed that DHKB possessed the highest identity with the LemA hybrid kinase from the bacteria, *Pseudomonas syringae* pv. *syringae* (Hrabak and Willis, 1992). The sequence identity, however, is only within the histidine kinase and the receiver domains and does not reveal any clues as to the sensor function of DHKB.

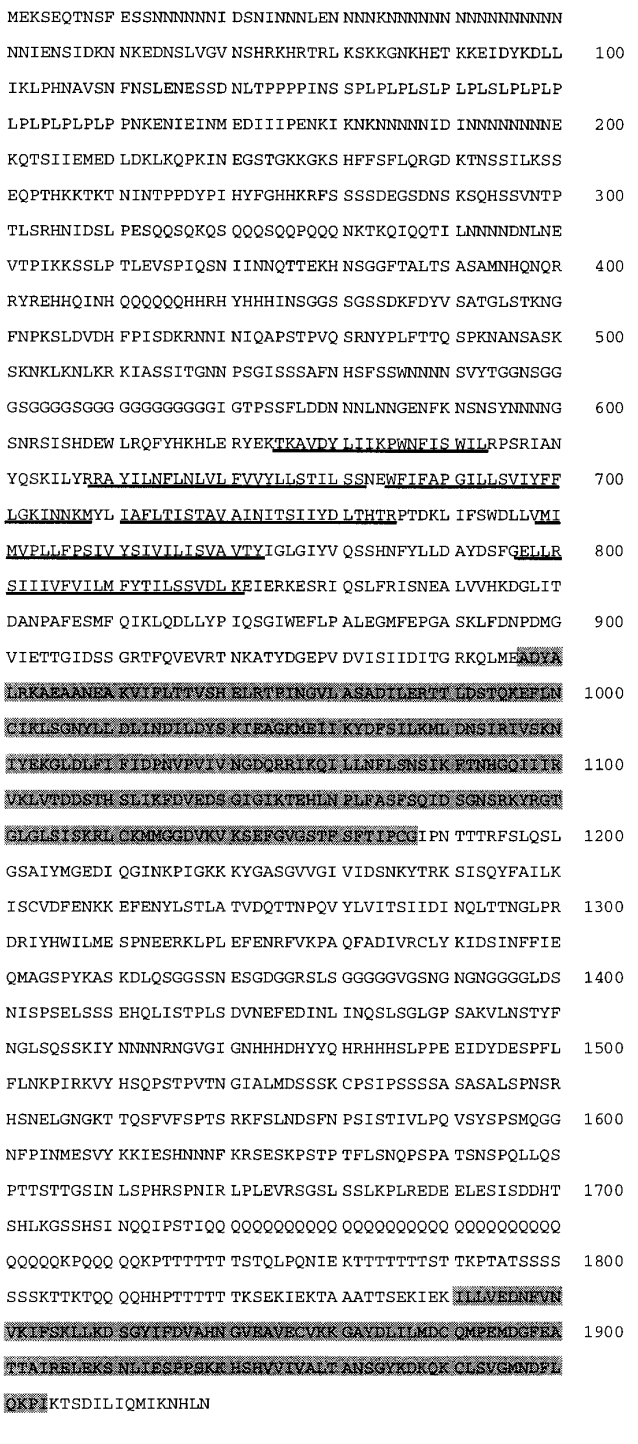

FIG. 1. Derived amino acid sequence of DHKB. The amino-terminal shaded region represents the histidine kinase domain. The carboxy-terminal shaded region represents the receiver domain. The underlined regions represent possible transmembrane-spanning regions.

The DHKB protein contains several long homopolymeric stretches of amino acids between the histidine kinase domain and the receiver domain and in the amino-terminal input domain (Fig. 1). These long stretches of a single amino acid were also noted in DHKA and DOKA, two other *Dictyostelium* hybrid histidine kinases (Schuster *et al.*, 1996; Wang *et al.*, 1996). The function of these repeats is unknown. However, the polyglutamine tract between the histidine kinase domain and the receiver domain may be acting as a flexible linker between modules (Wootton and Drummond, 1989). Analysis using Kyte–Doolittle and Hopp–Woods algorithms revealed five to six hydrophobic stretches of 17 to 23 amino acids in the amino terminus of DHKB (underlined in Fig. 1). This suggests that DHKB is likely a transmembrane-spanning sensor kinase; however, experimental localization is necessary to confirm this.

Expression of dhkB during growth and development. Northern analysis failed to detect the messenger RNA from *dhkB*, indicating a low abundance transcript. Hence, reverse transcriptase–PCR was used to determine the expression pattern of *dhkB* during growth and development of *Dictyostelium* (Fig. 3). *dhkB* mRNA was present in growing cells and increased after development was initiated. mRNA levels peaked around 12 h at the tipped aggregate stage, and the mRNA remained at the higher level throughout the remainder of development.

Disruption of dhkB. The *dhkB* gene was disrupted in order to examine its role during the growth and development of *Dictyostelium*. The entire histidine kinase domain of *dhkB* was removed, including the conserved histidine residue that has been shown to be necessary for the function of the kinase domain, and it was replaced with a Blasticidin S resistance cassette (Sutoh, 1993) to give p2C3/Bsr. KAx3 cells were transformed with the linearized construct, and individual Blasticidin S-resistant colonies were analyzed for disruption of the *dhkB* gene. Southern blot analysis revealed several Blasticidin-resistant cell lines that lacked the histidine kinase domain of *dhkB* and had in its place the Blasticidin-resistance gene (data not shown). Two *dhkB* null strains, each from a separate transformation experiment, were designated 24A and 47B and were used in all subsequent experiments.

The *dhkB*-null strains grew normally on bacterial lawns. As others have found that osmosensing is a frequent function of histidine kinases in eukaryotes, the response to alterations in osmolarity was examined. The *dhkB*-null cells were subjected to either high (400 mM sorbitol) or low (ddH_2O) osmostress for 2 h followed by a determination of the number of surviving cells. In both instances, the number of viable $dhkB^-$ cells was the same as that for the $dhkB^+$ control, demonstrating the *dhkB*-null cells responded normally to osmostress, including undergoing typical cell shape changes that accompany changes in osmolarity (data not shown). Additionally, hyperosmotic conditions did not alter the development of *dhkB*-null cells (data not shown).

The *dhkB*-null strains developed with normal timing and formed a fruiting body with a few abnormalities (Fig. 4).

A

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                                  H Motif
                                     •
DHKD        LRSLYEKIKE LDELKTQFFX NVSHELRTPL ALIVGPTDKL LKDENVDINV
DHKC        RTSNLSIESV LNNKSIDMIS HLSHELRTPI HSVIASIQLF RSTI-LTVTQ
DHKB        ADYALRKAEA ANEAKVIFLT TVSHELRTPI NGVLASADIL ERTT-LDSTQ
DOKA        YRQAEMAAIE ATRLKTQFIT NISHELRTPC HGIVGMSQLL LDSQ-LTNTQ
DHKA        ILEKE-AAEE ANKAKSAFVA TVSHEVRTPL SGVIGVSDLL LETN-LSEEQ
LemA        LDLARKEALE ASRIKSEFLA NMSHEIRTPL NGILGFTHLL QKSE-LTPRQ
Etr1        LDLARREAET AIRARNDFLA VMNHEMRTPM HAIIALSSLL QETE-LTPEQ
Consensus   ...A...A.E A...K..F.. ..SHELRTP. .GI.G...LL ..T.-LT..Q

DHKD        RKDLEIVARN ARGLLKIVNN LLDISRLEAG KMNLNYSMVN LGQXAHLIAS
DHKC        NEYLSIIDTS ANTLLELVSN VLDYKRIRSG KLTLNNVDFN LCHVIEDVCA
DHKB        KEFLNCIKLS GNYLLDLIND ILDYSKIEAG KMEIIKYDFS ILKMLDNSIR
DOKA        RDNIDSIKRS TDSLISLIND ILDFSKLEYG KVTLENESFE LLPMIEEVLD
DHKA        RDYVQTIQKS SQALLTIIND ILDYSKLESR QLKMETLPFS IIETCQAVIH
LemA        FDYLGTIEKS ADNLLSIINE ILDFSKIEAG KLVLDNIPFN LRDLLQDTLT
Etr1        RLMVETILKS SNLLATLMND VLDLSRLEDG SLQLELGTFN LHTLFREVLN
Consensus   R..L..I..S ...LL.LIND ILD.SKLE.G KL.L....FN L......V..

                                                    N Motif
DHKD        CFEILAREKS LDF---SII- --TPSEPMMA AIDADKMQRV ITNLISNAFK
DHKC        MVSPQAQAKS LQIASFIFI- --H--CPLSF YGDPIRLRQV LLNLIGNGLK
DHKB        IVSKNIYEKG LDL--FIFI- --DPNVPVIV NGDQRRIKQI LLNFLSNSIK
DOKA        SQATAANRKG IDL---IFVM GRDYPVPPVI FGDRNSLKKV LLNLVGNAVK
DHKA        MLSVAAND-D VDI--LLRV- --PPNVPRII FGDAMRMRQV LLNRLSNAIK
LemA        ILAPAAHAKQ LEL--VSLV- --YRDTPLAL SGDPLRLRQI LTNLVSNAIK
Etr1        LIKPIAVVKK LPI--TLNL- --APDLPEFV VGDEKRLMQI ILNIVGNAVK
Consensus   .....A..K. LD.--....- --.P..P... .GD..RL.QV LLNL.SNA.K

                                                    G1 Motif
DHKD        FTPSGGAGK- ----CILEKF -----DLSPN KPGFQIVVSD TGPGIPDNLH
DHKC        YTNKGQVCI- ----SVEPE- -----QVNEH CMYLHFQVKD SGIGIKEENM
DHKB        FTNHGQIII- ----RVKLV- -----TDDST HSLIKFDVED SGIGIKTEHL
DOKA        FTETGFVLL- ----EISTD- -----YESGD QISLRFTVKD SGIGIPENKI
DHKA        FTSRGHVLT- ----DISVDD SIPPTNTEEE IIHLCITIED TGIGIPQSLF
LemA        FTREGTIVAR AMLEDETEEH AQ--------- ---LRISVQD TGIGLSSQDV
Etr1        FSKQGSISVT ALVTKSDTRA ADFFVVPTGS HFYLRVKVKD SGAGINPQDI
Consensus   FT..G....- ----..... ----------- ...L...V.D SGIGI.....

            F Motif              G2 Motif
DHKD        EIIFERFRQV DGS-STRKHG GTGLGLSIVK EFVTLHGG-T VTIHNISTGG
DHKC        SKLFAGFSQV NNGGTTQEAL GSGLGLAISK DLVELMGG-K IWCSSNATQN
DHKB        NPLFASFSQI DSG-NSRKYR GTGLGLSISK RLCKMMGG-D VKVKSEFGVG
DOKA        EQIFVPFGQI DGS-FSRKYG GSGLGLSFCK ELVALMGG-Y IRVESGDQEG
DHKA        DSIFEPPFSQA DNS-TTRKYG GTGLGLSITK RLIEEVMGGT IQVSSIVGQG
LemA        RALFQAFSQA DNS-LSRQPG GTGLGLVISK RLIEQMGGEI GVDSTP-GEG
Etr1        PKIFTKFAQT QSL-ATRSSG GSGLGLAISK RFVNLMEGNI WIESDGLGKG
Consensus   ..IF..FSQ. D.S-.TRK.G GTGLGLSISK RLV.LMGG-. ...SS..G.G

DHKD        AQFTLRLPLT
DHKC        NGEAGCTFHF
DHKB        STFSFTIPCG
DOKA        GKGTTFWFAI
DHKA        SKFK-CIIP-
LemA        SEFWISLKLP
Etr1        CTAIFDVKL-
Consensus   ..........
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B

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                                                    D Motif
                                                       •
Etr1        VLVMDENGVS RMVTKGLLVH LGCEVTTVSS NEECLRVVSH --EHKVVFMD
DHKB        ILLVEDNFVN VKIFSKLLKD SGYIFDVAHN GVEAVECVKK G-AYDLILMD
DOKA        ILVAEDNDIN IKVVVRQLEK LGYTAIVGIN GLKALEIIGS F-PICLILLD
DHKA        ALIVEDNELN RKVLAQLFKK IDWTISFAEN GREALKEITG ERCFDIVFMD
LemA        VLCVDDNPAN LLLVQTLLED MGAEVVAVEG GYAAVNAVQQ E-AFDLVLMD
DHKC        IMILDDNPVS LKLMQRILES RGFECYPFDC SEKAVAQLDQ V-NPAIIFMD
DHKD        VLVVEDNPEM NRFIAELLSK Y-YFVVTAFD GVEGIEKTRA I-TPDLIVTD
Consensus   .L.VEDN..N .K....LL.. .G........ G.EA...... .-..DLI.MD

Etr1        VCMPGVENYQ IALRIHEKFT KQ-----RHQ R---PLLVAL SGNTDKSTKE
DHKB        CQMPEMDGFE ATTAIRELEK SNLIESPPSK KHSHVVIVAL TANSGYKDKQ
DOKA        CQMPQMDGFT CSTILRQIE- ------PTGQ R---IPIIAM TANDS---KD
DHKA        CQMPVLDGFQ TTKIIRSKER EN-----NWK R---MNIVAL SAGSSSSFVQ
LemA        VQMPGMDGRQ ATEAIRAWEA ER-----NQS S---LPIVAL TAHAMANEKR
DHKC        CEMPKMNGXE CTQLIRKREQ ESLCL--LKD X---KIIIAL TAHINPEIQV
DHKD        CMMPRMSGDE MVEQLRSDEQ --------FD N---IPILLL TAKADENLRI
Consensus   CQMP.MDG. .T..IR..E. ..-----... .---..IVAL TA......K.

                    K Motif
Etr1        KCMSFGLDGV LLKPV
DHKB        KCLSVGMNDF LQKPI
DOKA        RCFEVGMDDY LSKPV
DHKA        DCLDSGMDSF MGKPI
LemA        SLLQSGMDDY LTKPI
DHKC        KCFDAGMNDF ISKPF
DHKD        KLLQNGVSDY VNKPF
Consensus   KCL..GMDD. L.KP.
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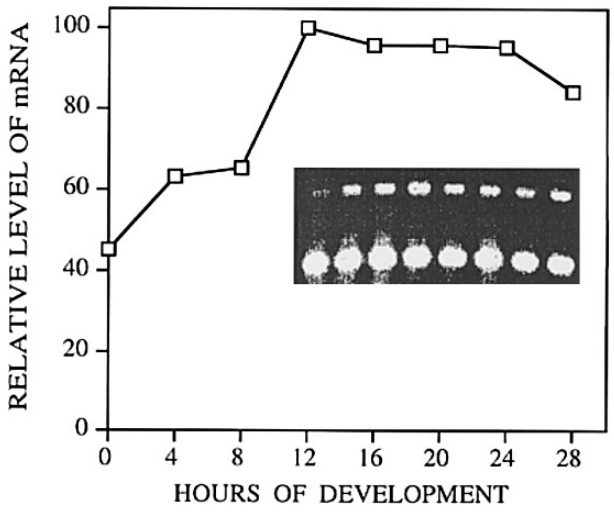

FIG. 3. *dhkB* mRNA expression during development. RT–PCR was used to examine the relative levels of mRNA from growing wild-type KAx3 cells and at various times (hours) during development. The inset shows the phosphorimage, with the upper band corresponding to *dhkB* mRNA and the lower band corresponding to the constitutively expressed H7 mRNA. The resulting bands were quantitated using a Molecular Dynamics Phosphorimager and normalized to the H7 internal control. The most intense *dhkB* band was set at 100%, and all other values are relative to this.

The *dhkB* null cells formed slugs that migrated normally and demonstrated normal phototaxis (data not shown). The fruiting bodies formed by null cells had slightly larger stalks than $dhkB^+$ fruits (Fig. 4). In addition, the sori of $dhkB^-$ fruiting bodies were glassy or transparent in appearance and had a darker yellow pigmentation in comparison to the $dhkB^+$ culminate (Fig. 4). In mature fruits examined after 3 days of development, less than 4% of the cells in the $dhkB^-$ sorus were mature spores as determined by visual inspection and challenge with heat and detergent. This indicated

FIG. 2. Amino acid sequence alignment of the DHKB histidine kinase and receiver domains. The DHKB histidine kinase domain (A) and receiver domain (B) were aligned with the DHKA (Wang *et al.*, 1996), DOKA (Schuster *et al.*, 1996), ETR1 (Chang *et al.*, 1993), LemA (Hrabak and Willis, 1992), DHKC (unpublished data), and DHKD (unpublished data) histidine kinase and receiver domains. (A) The H motif represents the conserved region containing the histidine kinase residue (closed circle) proposed to be autophosphorylated. The N and F motifs are conserved regions of unknown function. The G1 and G2 motifs are conserved regions involved in binding ATP. The primers used in the original degenerate PCR reaction are indicated by the arrows. (B) The D motif contains a conserved aspartate (closed circle) that receives the phosphate from the histidine kinase domain. The K motif contains a conserved lysine residue. The consensus sequence is indicated below the alignments and was determined with a 50% cutoff value.

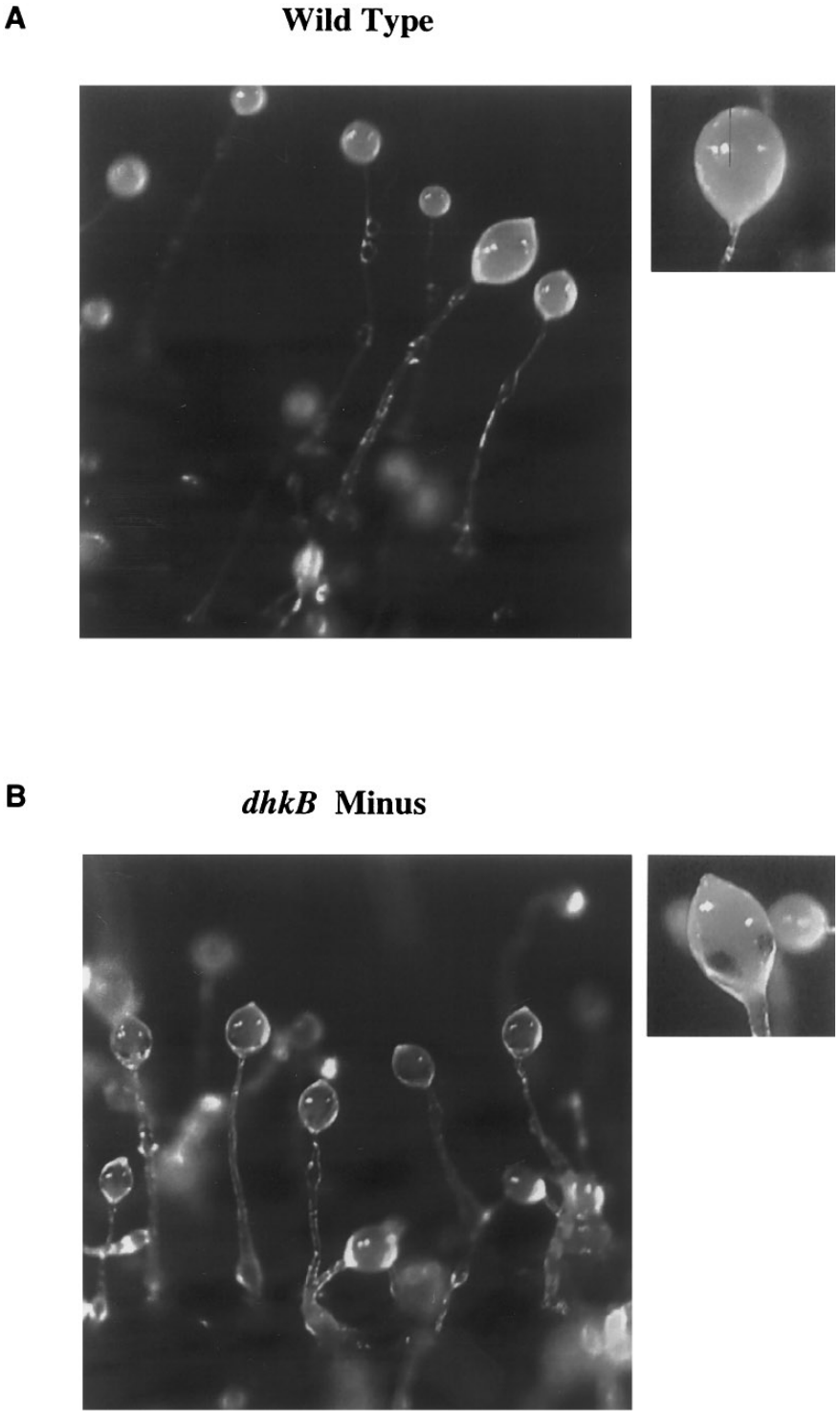

FIG. 4. Phenotype of the *dhkB*-null strains. Fruiting bodies formed from cells of *dhkB*$^-$ and *dhkB*$^+$ strains were photographed 48 h after being plated for synchronous development. (A) Parental strain, KAx3; (B) *dhkB*$^-$ strain 24A.

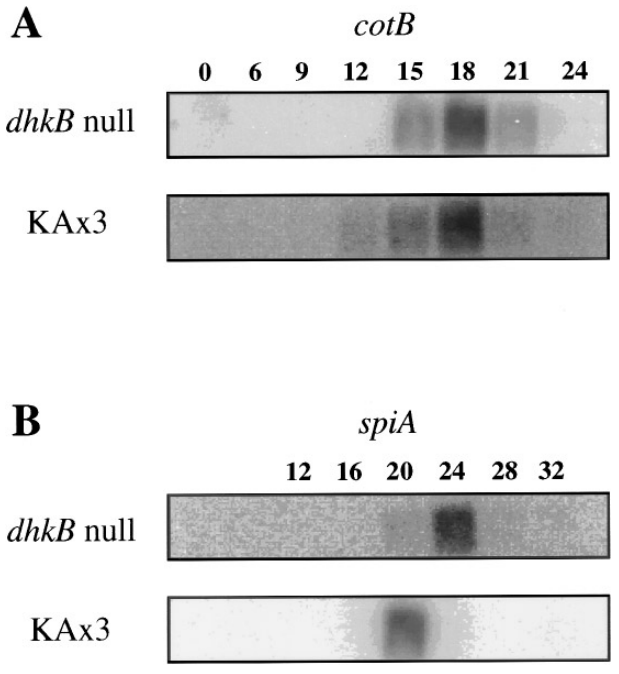

FIG. 5. Northern analysis of mRNA from *dhkB*$^+$ and *dhkB*$^-$ strains during development. Total RNA was isolated from growing cells and at the times indicated (in hours) after the onset of development. The filters were probed for *cotB* (A) or *spiA* (B) mRNA, followed by washing and visualization of hybridization using a phophorimager.

that DHKB may be involved in the terminal differentiation of prespore to spore cells.

Developmental gene expression. To more precisely determine the effects of *dhkB* on cellular differentiation, the expression of cell-type-specific markers was investigated. RNA was isolated from *dhkB*-null cells at various times after the initiation of development and was subjected to Northern analysis. The expression profile of the prestalk-specific marker *ecmA* (Jermyn *et al.*, 1987) was normal in the *dhkB*-null strains (data not shown). The mRNAs for the prespore-specific marker *cotB* (Fosnaugh and Loomis, 1993) and the spore-specific marker *spiA* (Richardson and Loomis, 1992; Richardson *et al.*, 1994) were expressed in *dhkB*-null cells in normal amounts. However, for both genes, the timing of expression was delayed approximately 2 to 4 h relative to the timing in *dhkB*$^+$ cells (Fig. 5). The expression of *spiA* indicated that terminal spore differentiation had been initiated. Therefore, if DHKB were involved in spore maturation, it must be during a late event.

Spore germination. Cells were collected from *dhkB*$^+$ and *dhkB*-null fruiting bodies at 22 h (late culmination), 29 and 48 h of development and viewed by phase-contrast microscopy to more closely examine the differentiating spores in the sorus. Mature spores have a morphology distinct from that of prespore cells; spores are visibly smaller with a characteristic ellipsoidal shape. Additionally, the synthesis of the middle cellulose layer of the spore coat is a late event in spore formation, and its presence indicates spore coat formation is complete or nearly so (West and Erdos, 1990). Thus, the fluorescent dye calcofluor, which stains cellulose, was used to confirm the presence of intact spore coats in *dhkB*-null spores.

At 22 h nearly all of the *dhkB*$^-$ cells (90%) in the rising spore mass had formed normal-appearing electron dense, ellipsoidal spores that stained strongly for cellulose, indicating the presence of a spore coat (Figs. 6C and 6D). The remaining cells were larger and amoeboid, probably representing cells that had not undergone sporulation as would be expected since all of the fruiting bodies were not at the exact same point in culmination. By 29 h the cell mass in all of the *dhkB*$^-$ culminates had reached the top of the stalk and normally would contain only mature spores as seen for the *dhkB*$^+$ control (Figs. 6A and 6B). However, most of the cells in the *dhkB*$^-$ sorus no longer appeared to be spores; instead they looked to be in the swelling stage of germination (round) or were amoeboid cells (Fig. 6E) (Raper, 1984). Several examples could be found of an amoeba emerging from a spore (arrows in Fig. 6E). The number of fluorescing cells had dropped substantially by this time with fewer than 33% of the cells in the sorus stained for cellulose. Of those that did stain, many appeared to be swollen spores during the early stages of germination (Fig. 6F).

At 48 h the number of cells in the sorus that stained for cellulose were still around 33% (Fig. 6H), yet very few ellipsoidal spores were observed (Fig. 6G). At this time, the majority of the emerged amoebae were significantly reduced in size relative to their appearance at 29 h. More than likely, this is due to the hyperosmotic conditions of the sorus (Cotter *et al.*, 1992). By 72 h the majority of the cells were very small or had apparently lysed after the prolonged exposure to a lack of nutrients and to the osmotic environment of the sorus. A few calcofluor-staining cells were still present at 72 h; however, photographs were not representative of the original number of cells in the sorus.

Together, these results indicate that mature or nearly mature spores were forming at the appropriate time within sori of *dhkB*$^-$ fruits. Initially, the *dhkB*$^-$ spores possessed the characteristic shape of spores and possessed an intact spore coat. However, the *dhkB*$^-$ spores began to germinate within the sorus around 24 h postinitiation of development; continued germination accounted for the low number of spores present in the sorus at 72 h instead of no spores ever being formed.

The cells in the sori were isolated from 25 to 72 h, counted, and plated with *Klebsiella pneumoniae* at various titers to determine their viability. After 4 to 7 days plaques were counted. All of the cells in the sorus were viable up to 29 h of development, whether they were spores, swollen spores, or emerged amoebae (Table 1). However, by 48 h the number of viable cells was only about 19%, slightly less than the number of calcofluor staining cells. This indicates that the emerging amoebae were dying in the environment

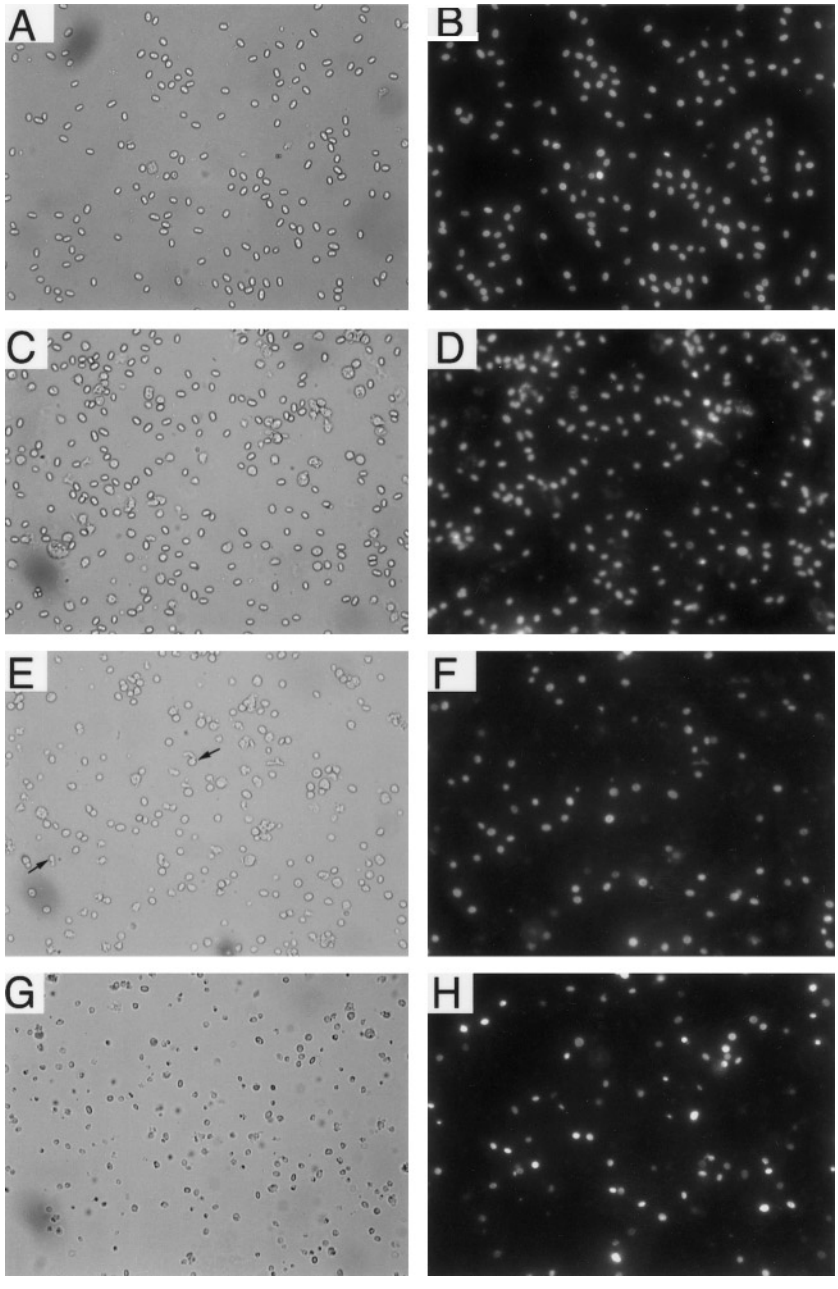

FIG. 6. Visualization of cells from the sori of *dhkB*$^-$ strains. Cells were collected from sori of *dhkB*$^+$ culminates and visualized by phase-contrast (A) and fluorescence (B) microscopy after staining with calcofluor. Cells were collected from sori of *dhkB* nulls at 22 h (C), 29 h (E), and 48 h (G) and examined by phase-contrast microscopy after calcofluor staining. The same cells were visualized by fluorescence microscopy at 22 h (D), 29 h (F), and 48 h (H). Arrows indicate emerging amoebae.

of the sorus. By 72 h the number of viable cells was slightly lower at 16%. This number is probably artificially elevated because many cells apparently had lysed by this time, resulting in a lower number of cells being collected from the 72-h sori of the *dhkB*$^-$ but not the *dhkB*$^+$ strain to which the numbers were compared.

The number of heat- and detergent-resistant spores in the sorus were determined as well. Amoebae and germinating spores are killed by heat and detergent challenge, whereas dormant spores are resistant and survive such a challenge (Hadwiger and Firtel, 1992; Kay, 1987). The number of viable *dhkB*$^-$ spores, after challenge with heat and detergent,

TABLE 1
Viability of Cells in the Sori of *dhkB* Null Cells

Hours of development	% Cell viability[a]	% Spore viability
25	—	23 ± 6
29	100	19 ± 4
48	19 ± 5	16 ± 1
72	16 ± 3[b]	3 ± 0.6
72[c]	—	48 ± 7

Note. Cells were isolated from the sori of *dhkB* nulls at various times after the initiation of development and assayed for the ability to grow without any prior treatment (cell viability) or after heat and detergent treatment (spore viability).

[a] Percentage relative to $dhkB^+$ viable cells or spores.

[b] Percentage may be elevated due to cell lysis by this time point (see Materials and Methods).

[c] Data from *dhkB* nulls transformed with SP60::PKAcat. The percentage given is relative to viable spores obtained from $dhkB^+$ cells transformed with SP60::PKAcat.

decreased over time (Table 1). However, as germination of $dhkB^-$ spores began to occur around 24 h and detergent resistance was not attained until 25–26 h for $dhkB^+$ spores (and presumably for nongerminating $dhkB^-$ spores), 100% resistance was not attained. By 72 h, the number of resistant spores had decreased to 3%, corresponding with the microscopy and fluorescence findings presented above.

In addition to germinating *in situ*, $dhkB^-$ spores germinated *in vitro* under conditions that prevent germination of wild-type spores. $dhkB^+$ and $dhkB^-$ spores were collected at 22 h and suspended in HL-5 medium at 10^8 per milliliter, a titer that normally prevents germination due to the high concentration of the germination inhibitor discadenine (Abe *et al.*, 1976). While less than 20% of the $dhkB^+$ spores germinated under these conditions, more than 80% of the $dhkB^-$ spores germinated. The emerged amoebae remained viable and did not shrink, supporting the conclusion above that loss of viability after emerging in the sorus was due to a lack of nutrients and high osmolarity.

Mixing experiments with 50% *dhkB*-null cells and 50% wild-type cells resulted in an intermediate number of mature spores produced after 72 h (data not shown). This indicated that the wild-type cells were not able to compensate for the defect in *dhkB* nulls, and thus the phenotype is cell-autonomous.

Suppression of germination by PKA. An active PKA has previously been implicated in inhibiting spore germination (Hopper *et al.*, 1995; Kay, 1989; Mann *et al.*, 1994; van Es *et al.*, 1996). Several experiments were performed to examine a potential link between DHKB and PKA. PKA can be activated ectopically by overexpression of the catalytic subunit (Mann *et al.*, 1994; Anjard *et al.*, 1992). $dhkB^+$ and $dhkB^-$ cells were transformed with SP60::PKAcat, a plasmid containing the gene for the catalytic subunit under the control of the prespore-specific promoter SP60 (Mann *et al.*, 1994). After 72 h of development, 48% of the spores from the sori of $dhkB^-$ cells transformed with SP60::PKAcat remained as viable spores (Table 1), indicating a significant suppression of the germination that occurred for $dhkB^-$ spores not ectopically expressing PKA cat (<4% viable spores at 72 h).

In addition, the cell permeable and phosphodiesterase resistant cAMP analog, 8-bromo-cAMP (Kay, 1989), was used to ectopically activate PKA in $dhkB^-$ spores. As shown in Fig. 7, such activation substantially inhibited germination of $dhkB^-$ spores. Finally, the intracellular levels of cAMP were found to be reduced in $dhkB^-$ spores relative to $dhkB^+$ spores. At 24 h postdevelopment, when most $dhkB^-$ spores have not yet germinated, the levels of intracellular cAMP in $dhkB^+$ spores were 2.4 times the levels in $dhkB^-$ spores: 1.22 ± 0.32 pmol/1.5×10^7 $dhkB^-$ spores, 2.93 ± 0.11 pmol/1.5×10^7 $dhkB^+$ spores ($P < 0.01$, $n = 3$, Student t test).

DISCUSSION

In the past several years, it has become clear that two-component systems control important regulatory pathways in not only prokaryotes but also eukaryotes. The sensor proteins identified in the eukaryotes *Neurospora crassa* (Alex *et al.*, 1996), *Arabidopsis thaliana* (Chang *et al.*, 1993), and *D. discoideum* (Schuster *et al.*, 1996; Shaulsky *et al.*, 1996; Wang *et al.*, 1996) have been shown to be involved in regulating aspects of development in these organisms. We identified three additional sensor proteins by using de-

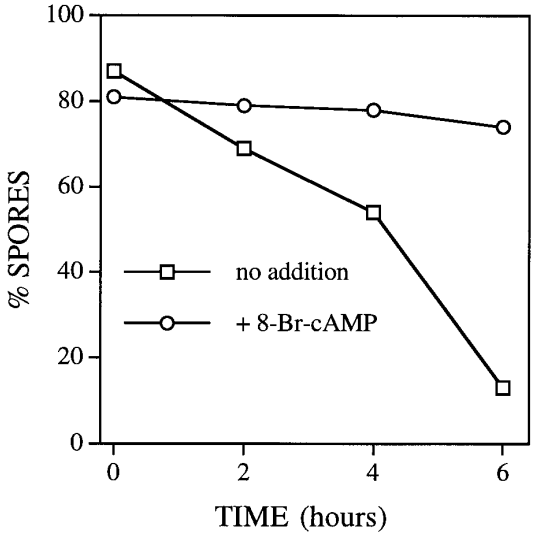

FIG. 7. Suppression of $dhkB^-$ spore germination by 8-bromo-cAMP. $dhkB^-$ spores were collected 24 h after plating cells for development. The spores were incubated at 10^7 per milliliter in HL-5 medium with (○) or without (□) 8-bromo-cAMP. The number of spores and emerged amoebae were determined, and the percentage of spores is plotted versus time in hours.

generate PCR to search for histidine kinase genes that may be involved in the development of *D. discoideum*. Three genes encoding putative sensor proteins were identified: *dhkB* described herein and *dhkC* and *dhkD* (unpublished results). Due to the high degree of identity with other eukaryotic and prokaryotic sensors it is probable that the conserved histidine of DHKB is autophosphorylated and the phosphate is transferred to an aspartate in the receiver domain. All of the sensors identified in eukaryotes have been hybrid histidine kinases, containing both the receiver and histidine kinase modules within the same protein, except the ERS gene in *A. thaliana* (Hua *et al.*, 1995). DHKB is another member of this growing family of eukaryotic hybrid histidine kinases.

The disruption of *dhkB* by removal of the histidine kinase domain resulted in cells that formed fruiting bodies that appear fairly normal except they have slightly thickened stalks and by 48 h of development the sorus has a glassy appearance and a darker yellow pigmentation compared to $dhkB^+$ fruiting bodies. The aberrant appearance of the sorus is due to precocious germination of *dhkB*-null spores into amoebae within the sorus. The *dhkB* message is present throughout growth and development, yet a lack of *dhkB* expression has little effect until late development and germination.

The *dhkB* mRNA peaks around the tipped aggregate stage of development when totipotent amoebae are differentiating into prestalk and prespore cells. In *dhkB*-null cells, the accumulation of the prespore gene *cotB* and the spore differentiation marker *spiA* are slightly delayed. Thus, DHKB is playing a role earlier in development than is indicated by the major morphological defect observed in *dhkB*-null strains. What significance this has on stalk formation and spore germination remains to be determined. The mRNA for *spiA* first appears during midculmination as prespore cells begin to differentiate into spore cells (Richardson *et al.*, 1991), and it is believed that *spiA* expression tracts the initial stages of prespore to spore differentiation (Richardson *et al.*, 1994). *spiA* is expressed during mid- to late-culmination in *dhkB*-null fruiting bodies, indicating that the signals linking spore formation with morphogenesis are functional and that the initiation and early stages of spore maturation are occurring. During spore formation the spore coat proteins are released from the prespore vesicles and assembled into the electron-dense spore coat (West and Erdos, 1990). These proteins make up the inner and outer layer of the spore coat, and after formation of these layers newly synthesized cellulose is deposited to generate the middle layer of the spore coat. By late-culmination the $dhkB^-$ prespore cells have released the spore coat proteins stored in the prespore vesicles and have assembled the electron-dense spore coat. The spore coat stains with calcofluor indicating the cellulose layer has also been produced. Additionally, the $dhkB^-$ spores dehydrate and acquire the characteristic ellipsoidal shape, resulting in spores which are indistinguishable from $dhkB^+$ mature spores based on these criteria.

In normal wild-type fruiting bodies the spores remain dormant in the sorus in response to several factors, including the germination autoinhibitor discadenine, high osmolarity, and the lack of activating nutrients or growth factors (Abe *et al.*, 1976; Ceccarini and Cohen, 1967; Cotter *et al.*, 1992). By 29 h of development ellipsoidal spores, swollen spores, and emerging nascent amoebae are evident in the *dhkB*-null sori. Thus, spores begin to germinate within the sorus of *dhkB*-null fruiting bodies soon after they have matured. This indicates that DHKB is normally active in spores, and inactivation of DHKB controls the initiation of spore germination.

The osmosensing two-component signal transduction system of *Saccharomyces cerevisiae* is the best understood of the eukaryotic systems (Posas *et al.*, 1996). The signal transduction circuit consists of an intricate two-component phosphorelay mechanism, first described in the *Bacillus subtilis* sporulation pathway (Burbulys *et al.*, 1991), that feeds into a MAP kinase cascade controlling the transcription of the general stress response genes (Posas *et al.*, 1996). The sensor kinase Sln1p of the yeast system and all but one of the two-component sensors identified in eukaryotes is a hybrid histidine kinase gene as is DHKB. It has been suggested that many of the eukaryotic hybrid kinases may be part of a phosphorelay (Appleby *et al.*, 1996; Posas *et al.*, 1996). The ethylene response pathway of *A. thaliana* involves at least two distinct sensor kinases (Chang *et al.*, 1993; Hua *et al.*, 1995). As with the yeast osmosensing system, the ethylene signaling system of *Arabidopsis* appears to converge with a MAP kinase cascade (Ecker, 1995).

The dual-specificity kinase SPLA of *Dictyostelium* shows significant sequence identity with the *Arabidopsis* CTR1 kinase involved in the ethylene response (Nuckolls *et al.*, 1996). Disruption of the *splA* gene results in a phenotype similar to that of *dhkB*-null cells. There is a slight delay in the appearance of *cotB* and *spiA* mRNA, the sorus is more translucent than in the wild-type, and the amoebae in the sori appear to lyse. The main difference is that *splA* nulls are believed to be involved in spore differentiation instead of germination (Nuckolls *et al.*, 1996). However, it is not clear whether the sori produced by $splA^-$ cells were examined prior to a time when precocious germination would have occurred. This leaves open the possibility that the SPLA kinase is a downstream component of the DHKB two-component pathway, thus tying the pathway to a possible MAP kinase cascade involved in controlling the initiation of germination.

We propose that DHKB is normally active in dormant spores, and the activated DHKB kinase results in the phosphorylation of a downstream response regulator which prevents its activation of the germination pathway (Fig. 8). The signal that activates DHKB may well be discadenine, the autoinhibitor of spore germination (Abe *et al.*, 1976; Ceccarini and Cohen, 1967), or some as yet unidentified inhibitor of germination. Discadenine is produced during late spore maturation and is localized to the spore matrix (Loomis, 1975). Its removal through dilution upon dispersal of the spores is required for the initiation of the germination pro-

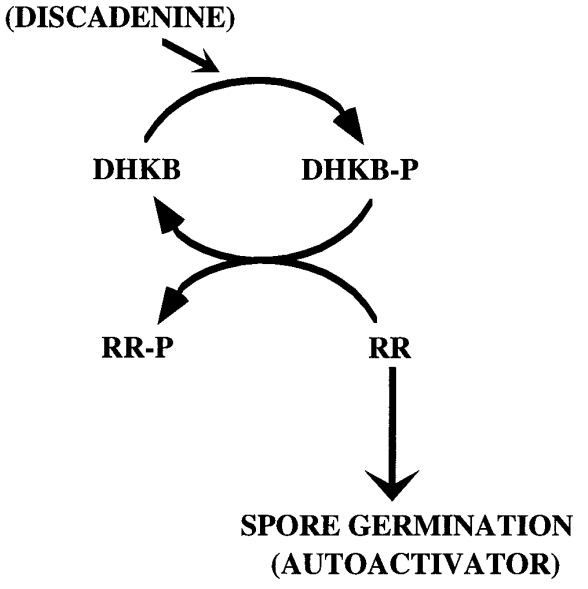

FIG. 8. A model of spore germination regulated by the DHKB signaling pathway. For details, see Discussion.

gram (Cotter, 1981; Cotter *et al.*, 1992). As indicated by the model, the absence of DHKB in the null strains prevents discadenine function and the spore germination pathway is thus initiated within the sorus. Because production of autoactivator is part of the normal germination program (Cotter, 1989), we propose that the DHKB signaling pathway also regulates autoactivator production. Further experiments are required to confirm that the DHKB pathway involves autoinhibitor and autoactivator.

To date, only one putative response regulator has been identified in *Dictyostelium*. The *regA* gene encodes a protein with an N-terminal receiver domain and a C-terminal cyclic nucleotide phosphodiesterase domain (Shaulsky *et al.*, 1996). It has been proposed that phosphorylation of REGA inactivates its phosphodiesterase activity which would result in an increase in intracellular cAMP. Increased cAMP concentrations activate PKA causing it to initiate and to regulate spore maturation (Hopper *et al.*, 1995; Kay, 1989; Mann and Firtel, 1993; Mann *et al.*, 1994; Simon *et al.*, 1989). From the phenotype of the *dhkB*-null strains, it seems clear that DHKB is not involved in controlling REGA activity during the initial stages of spore maturation, and it has been proposed that another histidine kinase, DHKA, is initially regulating REGA in response to signals from prestalk/stalk cells (Shaulsky *et al.*, 1996).

In addition to its function in spore maturation, it has been found that spores derived from *regA*-null cells have significantly reduced germination efficiency (P. Schaap, personal communication). This would be predicted from our model if REGA were the downstream response regulator of DHKB. Once discadenine is produced during late spore maturation, DHKB would be activated and thus ensure that REGA remained inactive by keeping it in the phosphorylated state. This would maintain the relatively high intracellular cAMP concentrations within mature spores, and thus PKA would remain active. Both high cAMP levels and an active PKA have been implicated as inhibitors of spore germination (Hopper *et al.*, 1995; Kay, 1989; Mann *et al.*, 1994); van Es *et al.*, 1996).

The scenario described above is consistent with our findings that ectopic expression or activation of PKA suppresses precocious germination of *dhkB*$^-$ spores. In addition, we find that intracellular levels of cAMP are lower in *dhkB*$^-$ spores, being about 40% of that in *dhkB*$^-$ spores. Although these findings implicate PKA as being downstream of DHKB, the proposed mediating role of REGA remains to be determined.

Recently, the spore-specific adenylyl cyclase (ACG) was shown to help maintain an active PKA in response to high osmolarity (van Es *et al.*, 1996). Our results indicate that DHKB, probably in response to the autoinhibitor discadenine, also is required for maintenance of an active PKA and hence the prevention of spore germination. *Acg*$^-$ spores, which retain a functional DHKB, do not germinate within the sorus, whereas *dhkB*$^-$ spores, which retain a functional ACG, do germinate while in the sorus very soon after they mature. This indicates that DHKB can maintain an active PKA and inhibit germination in the absence of ACG but ACG alone is insufficient to maintain PKA activity and germination inhibition. These two signaling mechanisms appear to respond independently to the two major endogenous inhibitors of spore germination (discadenine and high osmolarity), while their outputs converge through PKA and presumably depend on the relative balance between cAMP synthesis by adenylyl cyclases and cAMP degradation by the phosphodiesterase activity of the postulated response regulator REGA.

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