Activated cAMP receptors switch encystation into sporulation

Yoshinori Kawabe^a, Takahiro Morio^b, John L. James^a, Alan R. Prescott^b, Yoshimasa Tanaka^b, and Pauline Schaap^{a,1}

^aCollege of Life Sciences, University of Dundee, Dundee, Angus, DD15EH, United Kingdom; and ^bGraduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki, 305-8572, Japan

Edited by Peter N. Devreotes, Johns Hopkins University School of Medicine, Baltimore, MD, and approved March 12, 2009 (received for review February 13, 2009)

Metazoan embryogenesis is controlled by a limited number of signaling modules that are used repetitively at successive developmental stages. The development of social amoebas shows similar reiterated use of cAMP-mediated signaling. In the model *Dictyostelium discoideum*, secreted cAMP acting on 4 cAMP receptors (cARs1–4) coordinates cell movement during aggregation and fruiting body formation, and induces the expression of aggregation and sporulation genes at consecutive developmental stages. To identify hierarchy in the multiple roles of cAMP, we investigated cAR heterogeneity and function across the social amoeba phylogeny. The gene duplications that yielded cARs 2–4 occurred late in evolution. Many species have only a cAR1 ortholog that duplicated independently in the Polysphondyliids and Acytostelids. Disruption of both cAR genes of *Polysphondylium pallidum* (*Ppal*) did not affect aggregation, but caused complete collapse of fruiting body morphogenesis. The stunted structures contained disorganized stalk cells, which supported a mass of cysts instead of spores; cAMP triggered spore gene expression in *Ppal*, but not in the cAR null mutant, explaining its sporulation defect. Encystation is the survival strategy of solitary amoebas, and lower taxa, like *Ppal*, can still encyst as single cells. Recent findings showed that intracellular cAMP accumulation suffices to trigger encystation, whereas it is a complementary requirement for sporulation. Combined, the data suggest that cAMP signaling in social amoebas evolved from cAMP-mediated encystation in solitary amoebas; cAMP secretion in aggregates prompted the starving cells to form spores and not cysts, and additionally organized fruiting body morphogenesis. cAMP-mediated aggregation was the most recent innovation.

developmental signaling | evolution of multicellularity | Dictyostelia | Amoebozoa

The evolution of novel morphological features is due to changes in the developmental signaling processes that shape these features. The number of different signals that shape complex embryos, such as mammals, is surprisingly limited because the same signals, such as members of the wingless/wnt, hedgehog, TGF- β , and FGF families, are used many times over at successive developmental stages (1–3). Also, the signals and their associated transduction pathways are deeply conserved in evolution, often having homologous roles in shaping the body plan of lower invertebrates. The first multicellular organisms most likely deployed preexisting signaling systems from their unicellular ancestors, which were used to find food or mates, or to evade stress. To fully understand developmental signaling, it is of fundamental importance to identify which protist signaling pathways were used, and how they were adapted and elaborated to generate the ever increasing complexity of multicellular organisms.

The dictyostelid social amoebas offer unique opportunities to address this issue. They are as genetically diverse as animals, but alternate a sophisticated program of multicellular morphogenesis with a free-living amoeboid lifestyle. A robust molecular phylogeny is available, showing subdivision of all known species into 4 groups, with the model *Dictyostelium discoideum* residing

in the most derived group 4 (4). During *D. discoideum* development, the deeply conserved intracellular messenger cAMP has multiple roles as a secreted signal, detected by 4 homologous cAMP receptors (cAR1–4) (5). cAMP pulses coordinate the aggregation of starving cells and organize the construction of fruiting bodies with a highly regulated pattern of spores and stalk cells. Secreted cAMP also up-regulates expression of aggregation genes, induces expression of spore genes, and inhibits stalk gene expression (6).

Single cAR genes were previously detected in 3 more basal dictyostelid taxa, but were only expressed after aggregation. The nonhydrolyzable cAMP analog SpcAMPS, which inhibits cAR-mediated pulsatile cAMP signaling, disorganized fruiting body formation in these taxa, suggesting an ancestral role for pulsatile cAMP signaling in fruiting body morphogenesis (7). However, it remains unresolved whether cARs also mediated gene expression in basal taxa, for which SpcAMPS is a normal agonist, and whether the detected cARs were unique or members of larger gene families.

To resolve these questions, we first mapped cAR heterogeneity in the Dictyostelia, showing multiple independent events of cAR gene duplication. Second, by successively disrupting all cAR genes of the early diverged taxon *Polysphondylium pallidum* (*Ppal*), we greatly expanded the opportunities for cAR functional analysis. Loss of 1 cAR caused reduced fruiting body branching, whereas loss of both disrupted fruiting body formation. Strikingly, without cARs, *Ppal* cells could not express spore genes, and formed cysts instead of spores in the stunted structures. Encystation is the major stress response of solitary protists, which is retained in several early diverging social amoebas. Our data show that cysts are ancestral to spores, and that activated cARs determine the choice between the 2 developmental pathways.

Results

cAR Heterogeneity in the Dictyostelia. *D. discoideum* has 4 homologous cARs with different functions in chemotaxis and gene regulation (5). To understand how these functions evolved, we first mapped patterns of cAR gene duplication across the Dictyostelid phylogeny. Two to 5 test species were selected from each of the 4 groups of Dictyostelia (Fig. 1A). Amplification of

Author contributions: Y.K., Y.T., and P.S. designed research; Y.K. and J.L.J. performed research; T.M. and A.R.P. contributed new reagents/analytic tools; Y.K. and P.S. analyzed data; and Y.K. and P.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. EU797651 (*DroscaAR3*), EU797652 (*DroscaAR4*), EU797653 (*DmucAR1*), EU797654 (*DmucAR2*), EU797655 (*DmucAR3*), EU797656 (*DmucAR4*), EU797657 (*PviocAR*), EU797658 (*DhracAR*), EU797668 (*PpalTasB*), EU797661 (*PpseTasA*), EU797662 (*PpseTasB*), EU797663 (*DgloTasA*), EU797664 (*DgloTasB*), EU797660 (*AanacAR*), EU797665 (*AsubcARE*), EU797666 (*AsubcARF*), EU797667 (*AsubcARG*), EU797659 (*DhifcAR*), and EU797669 (*DaurcAR*)].

¹To whom correspondence should be addressed. E-mail: p.schaap@dundee.ac.uk.

This article contains supporting information online at www.pnas.org/cgi/content/full/0901617106/DCSupplemental.

Fig. 1. Identification and phylogeny of cAMP receptors. (A) Species selection. The published SSU rRNA based phylogeny of all known Dictyostelia (4) is schematically represented. The position of species from each of the 4 major taxon groups that were selected for cAR identification are indicated. (B) Identification and phylogeny of cARs. Partial 324–330-bp cAR sequences were amplified by PCR from genomic DNAs of the test species. Complete coding sequences for *PpalTasB* and *DaurcAR* were obtained by inverse PCR with primers complementary to the first PCR product. All derived partial and complete cAR amino acid sequences were aligned with previously published cAR sequences, the *Ddis* cAR-like receptor *CrIA* and with cAR homologs in nondictyostelid organisms. Phylogenetic relationships between sequences were determined by Bayesian inference (36), from 108 (PCR products) to 253 (full-length protein) well-aligned positions. The Bayesian inference posterior probabilities of nodes are indicated by line thickness. Numerical values are displayed in Fig. S2. The tree was rooted on *DdisCrIA* and the 3 nondictyostelid receptors. GenBank accession numbers for published sequences are: *Arabidopsis thaliana* (Atha)GPCR: NP.175261; *Physcomitrella patens* (Ppat)GPCR: XP.001765654; *Danio rerio* (Drer)GPCR: XP.001332705; *DdiscAR1*: P13773; *DdiscAR2*: AAB25436; *DdiscAR3*: AAB25437; *DdiscAR4*: Q9TX43; *DmincAR*: AA559250, *PpaltasA*: BAA99285; *DfascAR*: AA559252.

cAR genes from genomic DNAs of the selected species yielded 1 or multiple products ranging from 324 to 330 bp. Their derived amino acid sequences shared 59–97% sequence identity between each other and *DdiscARs* (Fig. S1A). Four *cAR* homologs each, were amplified from the group 4 species *Dictyostelium mucoroides* (*Dmuc*) and *Dictyostelium rosarium* (*Dros*). *Ppal* and 2 related Group 2 species, excluding the *Acytostelids*, yielded 2 *cAR* homologs each. *Acytostelium subglobosum* (*Asub*) yielded 3 *cAR* homologs, but only a single *cAR* was isolated from *Acytostelium anastomosans* (*Aana*). All tested Group 1 and 3 species, and the group-intermediate species *Polysphondylium violaceum* (*Pvio*) contained only a single *cAR* homolog.

We next isolated full-length *cAR* genes by inverse PCR from at least 1 species in each group. Full-length sequences were already available for *DdiscARs* 1–4 in group 4 (5), *Dictyostelium minutum* (*Dmin*) *cAR* in group 3 (7), and 1 *Ppal* *cAR*, named *TasA* in group 2 (8). Complete coding regions were amplified for the *Dictyostelium aureo-stipes* (*Daur*) *cAR* in group 1, and for the second *cAR* of *Ppal*, which was named *TasB*. Alignment of the deduced amino acid sequences of *DaurcAR* and *PpalTasB* with other complete *cARs* shows that the regions that contain the 7 transmembrane helices are highly conserved, whereas the carboxyl-termini of the *cARs* are very diverse (Fig. S1B).

An alignment of all PCR products and full-length *cARs* was used to assess phylogenetic relationships between the *cARs*. Fig. 1B shows that the 4 *cARs* of *Dmuc* and *Dros* are most similar to *cARs* 1–4 in *Ddis*. The 2 *cARs* that were identified in *Dictyostelium gloeosporum* (*Dglo*) and *Polysphondylium pseudocandidum* (*Ppse*), 2 species that are closely related to *Ppal* (Fig. 1A), are

most similar *Ppal* *TasA* or *TasB*. The 3 *Asub* *cARs* are quite diverse, but still more similar to *cARs* than to the *Ddis* *cAR*-like protein *CrIA*. All nongroup 4 *cARs* are more similar to *DdiscAR1* than to *DdiscARs* 2–4. These data indicate that the gene duplications that gave rise to *cARs* 2–4 occurred in Group 4 taxa, and that independent *cAR* gene duplications occurred at least 3 more times.

Expression Patterns of *cARs* in *Ppal*. We planned to use the large genomic fragments of the *Dmin* and *DaurcARs* and *PpalTasB* to generate constructs for expression analysis and gene disruption. However, we have not been able to achieve stable transformation of *Dmin* and *Daur* cells yet. *Ppal* can be transformed; therefore, we concentrated on analyzing *cAR* function in this species. We first compared the temporal expression patterns of *TasB* and *TasA*. Fig. 2A shows that *TasB* expression is first visible after 2 h of starvation, to reach a plateau at 8 h when aggregation is completed. *TasA* mRNA first appears after aggregates have formed. Both genes remain highly expressed until late fruiting body formation. To visualize the spatial pattern of *TasB* expression, its 2.3-kb promoter was fused to the *LacZ* reporter gene and transformed into *Ppal* cells. Developing structures were incubated with X-Gal to visualize β -galactosidase activity. *TasB* was first expressed weakly at the center of streaming aggregates (Fig. 2B). Expression then increased throughout the newly formed sorogen, but soon appeared strongest at the tip region (Fig. 2C and D). *Ppal* fruiting bodies form whorls of side branches from cell masses that pinch off from the rear of the sorogen. *TasB* showed high expression at the site of separation of these cell

features that are shared by *tasA⁺tasB⁻* spores and cysts, but not by wild-type spores. Spores of all Dictyostelia can be lyophilized for storage (11), but this trait has not been demonstrated for cysts. We measured viability after lyophilization of wild-type spores and cysts, *tasA⁺tasB⁻* “spores” and *tasA⁺tasB⁻/TasB* spores. Fig. 5D shows that, in contrast to wild-type spores, cysts and *tasA⁺tasB⁻* spores completely lose viability after lyophilization. The *tasA⁺tasB⁻/TasB* spores are again fully resistant to lyophilization. Combined, these data show that *tasA⁺tasB⁻* spores are actually cysts.

To test this hypothesis directly, we investigated whether prespore differentiation is induced by cAMP in *Ppal* wild-type cells, but not *tasA*⁻*tasB*⁻ mutants, using the *Ppal* spore coat gene *SP45* (16) as a marker. Fig. 6 shows that cAMP induces *SP45* transcription in wild-type *Ppal*, but not in *tasA*⁻*tasB*⁻ cells. These data indicate that, in *Ppal*, cARs are essential for prespore induction by cAMP.

A *cAR1* Ortholog Duplicated Several Times During Dictyostelid Evolution. The model species *Ddis* has 4 *cAR* genes, and orthologs of all 4 genes were found in 2 other group 4 taxa, *Dmuc* and *Dros*

tion of the *tasA⁻tasB⁻* spores revealed that they have the same morphology as wild-type cysts, and are dissimilar to wild-type spores (Fig. 5 A–C; Fig. S4). We also sought for physiological

(Table S1), using religated *Clal* digested *Daur* genomic (g)DNA as template. The 1308-bp coding region of *Ppa/TasB* with 2515-bp 5' UTR and 482-bp 3' UTR, was obtained from 2 inverse PCR reactions using primer pairs *TasBlnv3/TasBlnv5* or *TasBlnvP1/TasBlnvP2* (Table S1) with religated *HpaI* or *EcoRV* digested *PpaI* gDNAs, respectively. All PCR products were subcloned in pBlue-scriptII (Stratagene) and sequenced.

DNA Constructs and Transformation

LoxP-Neo vector. G418 (neomycin) resistance is the only selectable marker for *PpaI*, and has to be recycled to create double knock-out mutants. A vector with *LoxP* excision sites flanking the actin6-neomycin cassette (A6neo) was constructed, which allows in vivo excision of the cassette by transient expression of Cre-recombinase (9). The *LoxP*-Bsr cassette of vector pLPBLP (9) was excised with *BamHI* and *HindIII*, and ligated into pUC19 to eliminate a second *XbaI* site in pLPBLP. The Bsr cassette was excised with *XbaI/EcoRV*, leaving the *LoxP* sites, those flanking 5' overhangs were filled in with KOD DNA polymerase (Toyobo). The actin6-NeoR cassette was excised with *EcoRI/HpaI* from pB105X (30), filled in, and blunt-end ligated into pUC19-*LoxP*, yielding vector pLoxNeo.

Vectors for *TasA* and *TasB* gene disruption. Partial *TasB* sequence with 0.8-kb 5' UTR and 2.2-kb 3' UTR was amplified by inverse PCR from *HpaI* digested and religated *PpaI* gDNA, using primers *TasBlnvK1* and *TasBlnvK2* (Table S1). The *BamHI* digested PCR product was cloned into *BamHI* digested pLoxNeo yielding *TasBloxP-KO*. To obtain a *TasA* disruption vector, partial *TasA* sequence with 1.4-kb 5' UTR and 1.1-kb 3' UTR was amplified with primers *TasAinvK1* and *TasAinvK2* from *XbaI* digested and religated *PpaI* gDNA, and cloned as above in pLoxNeo, yielding *TasAloxP-KO*. Cells were transformed as described previously (31), and screened for homologous recombination by 2 separate PCR reactions and analysis of Southern blots (for detailed procedures, see Fig. S5 and Fig. S6). To remove the A6neo cassette, knock-out cells were transformed with pA15NLS.Cre for transient expression of Cre-recombinase. Transformed clones were replica-plated onto autoclaved *Kaer* on LP agar with and without 200 μ g/mL G418 for negative selection.

***TasB* expression vector.** To express *TasB* from its own promoter, the *TasB* coding sequence and promoter were amplified separately by PCR, sequentially cloned into vector pEXP5 (32), and introduced into *tasA⁻tasB⁻neo⁻* cells (Fig. S3).

***TasB* promoter-LacZ construct.** The *TasB* promoter was amplified from *PpaI* gDNA using primers *TasBpro5'* and *TasBpro3'*, which contain *XbaI* and *BamHI* restriction sites respectively (Table S1). After digestion, the 2.3-kb PCR product was ligated into *BglIII/XbaI* digested vector pDGal17 vector (33), yielding vector *TasB::gal*.

RNA isolation and analysis. Total RNA was transferred to nylon membranes (7) and hybridized at 65 °C to [³²P] dATP-labeled *Sp45*, *TasA*, and *TasB* DNA probes. Hybridization of *PpaI* mRNA to a *Ddis* 1G7 probe was at 55 °C. *PpaI* *TasA* and *TasB* probes were prepared by PCR amplification of 152- and 192-bp sections, respectively, of the extreme 3' coding region of each gene using primer pairs *TasA-52/TasA-32* and *TasB-55/TasB-5* (Table S1) with vectors *TasAloxP-KO* and *pTasBexp* as templates. An 976-bp *Sp45* probe was amplified from *PpaI* gDNA using primers *Sp45F* and *Sp45R*.

Phylogenetic Analysis

Alignment. Deduced amino acid sequences of partial and full-length cAR genes, *DdisCrIA*, and nondictyostelid G protein coupled receptors (GPCRs) with the pfam05462 Dicty_CAR domain (34) were aligned with CLUSTALW (35), using the region spanning the first 6 transmembrane helices. The cARs aligned unambiguously, but the alignment of *DdisCrIA* and nondictyostelid GPCRs was edited according to their individual alignment to the pfam05462 domain.

Phylogeny. A cAR phylogeny was constructed by Bayesian inference (36). A mixed amino acid model was used with rate variation across sites estimated by a gamma distribution with 6 rate categories and no invariable sites. The analysis was run for 10⁶ generations, at which point the average SD of split frequencies was 0.0074. Posterior probabilities were averaged over the final 75% of trees. The analysis was also run with other prior settings such as introduction of a proportion of invariable sites, reducing rate categories to 4, or setting rate variation to equal. These changes did not affect tree topology.

ACKNOWLEDGMENTS. We thank Drs. Jan Faix (Ludwig Maximilians University, Munich) and Lisa Kreppel and Alan Kimmel (National Institutes of Health, Bethesda, MD) for their kind gifts of vectors pLPBLP and pA15NLS.cre. This work was supported by Biotechnology and Biological Sciences Research Council Grant BB/D013453/1 and Wellcome Trust Grant 076618.

- True JR, Carroll SB (2002) Gene co-option in physiological and morphological evolution. *Annu Rev Cell Dev Biol* 18:53–80.
- Guder C, et al. (2006) The Wnt code: Cnidarians signal the way. *Oncogene* 25:7450–7460.
- Varjosalo M, Taipale J (2008) Hedgehog: Functions and mechanisms. *Gene Dev* 22:2454–2472.
- Schaap P, et al. (2006) Molecular phylogeny and evolution of morphology in the social amoebas. *Science* 314:661–663.
- Parent CA, Devreotes PN (1996) Molecular genetics of signal transduction in Dictyostelium. *Annu Rev Biochem* 65:411–440.
- Aubry L, Firtel R (1999) Integration of signaling networks that regulate Dictyostelium differentiation. *Ann Rev Cell Dev Biol* 15:469–517.
- Alvarez-Curto E, et al. (2005) Evolutionary origin of cAMP-based chemoattraction in the social amoebae. *Proc Natl Acad Sci USA* 102:6385–6390.
- Kawabe Y, Kuwayama H, Morio T, Urushihara H, Tanaka Y (2002) A putative serpentine receptor gene *tasA* required for normal morphogenesis of primary stalk and branch structure in *Polysphondylium pallidum*. *Gene* 285:291–299.
- Faix J, Kreppel L, Shaulsky G, Schleicher M, Kimmel AR (2004) A rapid and efficient method to generate multiple gene disruptions in Dictyostelium discoideum using a single selectable marker and the Cre-loxP system. *Nucleic Acids Res* 32:e143.
- Hohl HR, Miura-Santo LY, Cotter DA (1970) Ultrastructural changes during formation and germination of microcysts in *Polysphondylium pallidum*, a cellular slime mould. *J Cell Sci* 7:285–306.
- Raper KB (1984) *The Dictyostelids* (Princeton Univ Press, Princeton).
- Ritchie AV, van Es S, Fouquet C, Schaap P (2008) From drought sensing to developmental control: Evolution of cyclic AMP signaling in social amoebas. *Mol Biol Evol* 25:2109–2118.
- Hopper NA, Harwood AJ, Bouzid S, Véron M, Williams JG (1993) Activation of the prespore and spore cell pathway of Dictyostelium differentiation by cAMP-dependent protein kinase and evidence for its upstream regulation by ammonia. *EMBO J* 12:2459–2466.
- Wang M, Van Driel R, Schaap P (1988) Cyclic AMP-phosphodiesterase induces dedifferentiation of prespore cells in Dictyostelium discoideum slugs: Evidence that cyclic AMP is the morphogenetic signal for prespore differentiation. *Development* 103:611–618.
- Anjard C, Zeng CJ, Loomis WF, Nellen W (1998) Signal transduction pathways leading to spore differentiation in Dictyostelium discoideum. *Dev Biol* 193:146–155.
- Gregg KY, Cox EC (2000) Spatial and temporal expression of a Polysphondylium spore-specific gene. *Dev Biol* 224:81–95.
- Schaap P, Konijn TM, Van Haastert PJM (1984) cAMP pulses coordinate morphogenetic movement during fruiting body formation of Dictyostelium minutum. *Proc Natl Acad Sci USA* 81:2122–2126.
- Schaap P (1985) cAMP relay during early culmination of Dictyostelium minutum. *Differentiation* 28:205–208.
- Siebert F, Weijer CJ (1992) Three-dimensional scroll waves organize Dictyostelium slugs. *Proc Natl Acad Sci USA* 89:6433–6437.
- Dormann D, Abe T, Weijer CJ, Williams J (2001) Inducible nuclear translocation of a STAT protein in Dictyostelium prespore cells: Implications for morphogenesis and cell-type regulation. *Development* 128:1081–1088.
- Alvarez-Curto E, et al. (2007) cAMP production by adenylyl cyclase G induces prespore differentiation in Dictyostelium slugs. *Development* 134:959–966.
- Van Es S, et al. (1996) Adenylyl cyclase G, an osmosensor controlling germination of Dictyostelium spores. *J Biol Chem* 271:23623–23625.
- Schaap P, Van Driel R (1985) Induction of post-aggregative differentiation in Dictyostelium discoideum by cAMP. Evidence of involvement of the cell surface cAMP receptor. *Exp Cell Res* 159:388–398.
- Van Haastert PJM, Konijn TM (1982) Signal transduction in the cellular slime molds. *Mol Cell Endocrinol* 26:1–17.
- Raizada MK, Murli CRK (1972) Transformation of trophic Hartmannella culbertsoni into viable cysts by cyclic 3'5'-adenosine monophosphate. *J Cell Biol* 52:743–748.
- Coppi A, Merali S, Eichinger D (2002) The enteric parasite Entamoeba uses an autocrine catecholamine system during differentiation into the infectious cyst stage. *J Biol Chem* 277:8083–8090.
- Lilly PJ, Devreotes PN (1995) Chemoattractant and GTPγS-mediated stimulation of adenylyl cyclase in Dictyostelium requires translocation of CRAC to membranes. *J Cell Biol* 129:1659–1665.
- Cornier FI, Lippincott CK, Masbad JJ, Parent CA (2005) The PI3K-mediated activation of CRAC independently regulates adenylyl cyclase activation and chemotaxis. *Curr Biol* 15:134–139.
- Lee S, et al. (2005) TOR complex 2 integrates cell movement during chemotaxis and signal relay in Dictyostelium. *Mol Biol Cell* 16:4572–4583.
- Nellen W, Firtel RA (1985) High-copy-number transformants and co-transformation in Dictyostelium. *Gene* 39:155–163.
- Kawabe Y, Enomoto T, Morio T, Urushihara H, Tanaka Y (1999) LbrA, a protein predicted to have a role in vesicle trafficking, is necessary for normal morphogenesis in Polysphondylium pallidum. *Gene* 239:75–79.
- Meima ME, Weening KE, Schaap P (2007) Vectors for expression of proteins with single or combinatorial fluorescent protein and tandem affinity purification tags in Dictyostelium. *Protein Expr Purif* 53:283–288.
- Harwood AJ, Drury L (1990) New vectors for expression of the *E. coli* lacZ gene in Dictyostelium. *Nucleic Acids Res* 18:4292.
- Bateman A, et al. (2004) The Pfam protein families database. *Nucleic Acids Res* 32:D138–D141.
- Chenna R, et al. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 31:3497–3500.
- Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.
- Dingermann T, et al. (1989) Optimization and in situ detection of Escherichia coli beta-galactosidase gene expression in Dictyostelium discoideum. *Gene* 85:353–362.