Check for updates

RESEARCH ARTICLE

REVISED NOVEL INVENTION OF SPORE INDUCTION IN A SISTER SPECIES TO GROUP 4 DICTYOSTELIA

[version 2; peer review: 3 approved]

Pauline Schaap

School of Life Sciences, University of Dundee School of Life Sciences, Dundee, Scotland, DD15EH, UK

v2 **First published:** 29 Oct 2024, **4**:239
https://doi.org/10.12688/openreseurope.18365.1
Latest published: 05 Dec 2024, **4**:239
https://doi.org/10.12688/openreseurope.18365.2

Abstract

Background

Dictyostelia are soil amoebas that aggregate to form fruiting bodies with spores and stalk cells in response to starvation. Where known, species across the dictyostelid phylogeny use secreted cAMP, detected by cAMP receptors (cARs) to induce the differentiation of spores and to organize fruiting body construction. However, recent deletion of the single *cAR* of *Polyspondylium violaceum (Pvio*) left both its fruiting bodies and spores intact.

Methods

To investigate whether *Pvio* sporulation can occur in the absence of secreted cAMP and to explore alternative inducers in a bioassay, three prespore genes were identified and gene fusions of their promoters with the *LacZ* reporter gene were transformed into *Pvio* cells. After assessing the spatial expression pattern of the genes and the stage at which prespore gene expression initiated, the effect of cAMP and other *Dictyostelium discoideum* (*Ddis*) signal molecules were tested on prespore gene expression *in vitro*.

Results

Pvio genes *g4562 (psp1)*, *g2696 (psp2)* and *g2380 (psp3)* were identified as homologs of *Ddis* spore coat genes. They were first expressed around 4 h of starvation in aggregation centres and later in the posterior 4/5th of emerging sorogens and the spore head of early fruiting bodies. Cells from dissociated 4 h aggregates and shaken in suspension for 6 h increased prespore-*LacZ* reporter activity 4-fold for

Open Peer Review

Approval Status ✓ ✓ ✓

	1	2	3
version 2 (revision) 05 Dec 2024		✓ view	
version 1 29 Oct 2024	✓ view	? view	✓ view

1. **Tamao Saito**, Sophia University, Tokyo, Japan
2. **Yusuke V. Morimoto**, Kyushu Institute of Technology, Fukuoka, Japan
3. **Shweta Saran**, Jawaharlal Nehru University, New Delhi, India

Any reports and responses or comments on the article can be found at the end of the article.

psp1 and 6-fold for *psp2,* but this increase was at least 5-fold higher when cells were plated on solid substratum for 6 h to develop normally. cAMP had no effect on prespore gene induction and neither had the *Pvio* chemoattractant glorin nor the *Ddis* chemoattractants and differentiation inducers folate, c-di-GMP, DIF-1, GABA, cGMP and 8Br-cAMP.

Conclusions

The *Pvio* lineage uniquely evolved a novel genetic network for synthesis, detection and processing of the signal that triggers its main survival strategy.

Plain language summary

The Dictyostelia are single-celled amoebas that eat soil bacteria. They aggregate to form a multicellular organism when food is depleted and construct a fruiting body with resilient spores, which become dispersed to new feeding grounds. Cyclic AMP (cAMP) plays a major role in this process as a secreted chemoattractant that brings the cells together and induces the expression of spore genes. cAMP also functions as an intracellular messenger for other signals that regulate the timely maturation of spores and stalk cells in the fruiting body.

The intracellular role of cAMP can be retraced to ancestors of the Dictyostelia, the solitary amoebas, which form less resilient cysts upon starvation. Here starvation and other stress conditions directly increase intracellular cAMP that then causes cells to encyst. All investigated Dictyostelia, including *Polyspondylium violaceum* that was studied here, use intracellular cAMP for spore and stalk cell maturation. However, *P. violaceum* is thus far the only species that can form fruiting bodies and spores without the receptors to detect cAMP. Does that mean that they also do not use secreted cAMP?

To answer that question a bioassay was developed to directly test effects of cAMP and other potential signal molecules on the expression of prespore genes. In the assay a low level of prespore gene expression was induced by an unknown signal that the cells themselves produced, but neither cAMP nor any of the six other signals that were tested had any effect on the expression of prespore genes. This means that *P. violaceum* evolved a whole set of novel genes to produce the unknown signal in a timely manner and to detect and process it. This is a major innovation in evolution and it will be of great interest to discover how this occurred and what the novel signal is.

Keywords

developmental network innovation, Polysphondylium violaceum, Dictyostelium discoideum, induction of sporulation, chemoattractant, cyclic AMP

This article is included in the Cell, Molecular and Structural Biology gateway.

This article is included in the Horizon 2020 gateway.

Corresponding author: Pauline Schaap (p.schaap@dundee.ac.uk)

Author roles: Schaap P: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Validation, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This research was funded by grant 742288 — ERC-2016-ADG from the European Research Council.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2024 Schaap P. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Schaap P. **NOVEL INVENTION OF SPORE INDUCTION IN A SISTER SPECIES TO GROUP 4 DICTYOSTELIA [version 2; peer review: 3 approved]** Open Research Europe 2024, **4**:239 https://doi.org/10.12688/openreseurope.18365.2

First published: 29 Oct 2024, **4**:239 https://doi.org/10.12688/openreseurope.18365.1

REVISED **Amendments from Version 1**

In response to reviewers 1 and 2's comments, the text "(compare black diamonds to coloured circles at 0 M)" was added to page 7 last sentence to explain how the roughly 4- to 6-fold increase in basal gene expression was deduced.

Following comment 1 of reviewer 2, the expression: "β-galactosidase activity was first observed in the centre of streaming aggregates" was changed into: "β-galactosidase activity was first observed inside newly formed aggregates with little to no activity in the inflowing streams".

The third top panel of Figure 2A was replaced by an image at higher magnification, arrows and text were inserted to indicate prespore, spore and stalk cells. The legend to Figure 2A was modified to incorporate these changes

In response to comment 3 of reviewer 2 the statement "The compounds were added in the concentration range where they are active in *Ddis*" is now followed up by the sentences: "These are 10^{-5} to 10^{-3} M for cAMP (Schaap & Van Driel, 1985), >10^{-10} M for DIF-1, 10^{-7} to 10^{-5} M for c-di-GMP, 10^{-9} to 10^{-4} M for GABA, 10 to 15 mM for 8Br-cAMP. Glorin, folate and cGMP were used in the range where cAMP induces prespore gene expression".

Any further responses from the reviewers can be found at the end of the article

Introduction

Dictyostelia evolved multicellularity independently from animals, plants and fungi within the single-celled Amoebozoa. They feed on bacteria but unlike their amoebozoan relatives, which survive starvation and other stressors as individual dormant cysts, dictyostelid amoebas aggregate instead to form fruiting bodies with dormant spores and up to three supporting cell types. Molecular phylogenetics subdivides Dictyostelia in four major and some minor groups (Schaap *et al.*, 2006; Schilde *et al.*, 2019). Group 4 contains the model organism *Dictyostelium discoideum* (*Ddis*) and displays several traits that set it apart from the other groups. Group 4 species uniquely use cyclic AMP (cAMP), secreted in pulses, as chemoattractant for aggregation. They mostly form large solitary fruiting bodies with spores supported by stalk, basal disc and upper and lower cup cells and have an intermediate migrating slug stage in which future (pre) spore and supporting cells initiate differentiation in appropriate proportions required for fruiting bodies construction. Species in groups 1–3 generally form smaller clustered and/or branched structures with at most two cell types, spores and stalk cells. Here most cells differentiate as prespore cells and transdifferentiate into stalk cells at the fruiting body tip. Species in these groups often use the dipeptide glorin as chemoattractant. Many group 1, 2 and 3 species can also still encyst individually when stressed, but this alternative life cycle is lost in group 4 (Romeralo *et al.*, 2013; Schilde *et al.*, 2014).

In addition to its role as chemoattractant, secreted cAMP acting on cAMP receptors (cARs) also induces the differentiation of prespore cells in group 4 (Schaap & Van Driel, 1985; Wang *et al.*, 1988), while intracellular cAMP acting on cAMP dependent protein kinase (PKA) induces the maturation of spores and stalk cells (Harwood *et al.*, 1992; Hopper *et al.*, 1993; Mann *et al.*, 1994). Evolutionary comparative studies showed that the roles of intracellular cAMP and PKA are conserved throughout Dictyostelia and are ancestrally derived from roles for cAMP and PKA as intermediates for stress-induced encystation in the solitary Amoebozoa (Du *et al.*, 2014; Funamoto *et al.*, 2003; Kawabe *et al.*, 2015; Ritchie *et al.*, 2008). While secreted cAMP pulses did not mediate aggregation in groups 1–3, they did organise fruiting body morphogenesis in the group 3 species *D. minutum* and the group 2 species *Polysphondylium pallidum (Ppal)* (Alvarez-Curto *et al.*, 2005; Schaap *et al.*, 1984). Secreted cAMP acting on cARs was also essential for induction of prespore differentiation in *P. pallidum* (Kawabe *et al.*, 2009).

These observations suggested an evolutionary scenario in which the roles of cAMP in spore and stalk maturation, induction of prespore differentiation, organisation of morphogenesis and finally in group 4 aggregation were sequentially derived from the ancestral role of cAMP as intermediate for stress-induced encystation (Schaap, 2016). Recent studies in *Polysphondylium violaceum (Pvio)*, which resides in a small sister clade to group 4, question the universality of this scenario. Unlike *Ppal,* where deletion of *car* genes disorganizes post-aggregative morphogenesis and prevents prespore and spore differentiation, deletion of the single *Pvio carA* gene leaves both its morphogenesis and spore differentiation intact (Kawabe & Schaap, 2023).

While it remains possible that a second *car* remained unsequenced in its draft genome (Narita *et al.*, 2020), lack of prespore markers made direct assessment of prespore gene induction by cAMP impossible. In this study, I identified relatives of three *Ddis* prespore genes in *Pvio*. *Pvio* cells transformed with gene fusions of the promoters of either gene and the *LacZ* reporter gene were used to study activation of the promoters by cAMP, glorin and signals known to promote or inhibit prespore differentiation in *Ddis*. The study indicated that the *Pvio* lineage invented a novel mechanism for prespore induction.

Methods

Cell culture

Polysphondylium violaceum strain QSvi11 (Kalla *et al.*, 2011) was grown in association with *Klebsiella aerogenes* on 1/3rd SM agar (13.9 g SM agar (catalogue number SMA0102 Formedium), 11 g agar (AGA03, Formedium), 1.27 g KH_2PO_4 (P5379, Sigma-Aldrich), 0.87 g $K_2HPO_4.3H_2O$ (P3786, Sigma-Aldrich) and 0.67 g $MgSO_4.7H_2O$ (230391, Sigma-Aldrich) per litre H_2O). Cells were harvested in PB (5 mM Na_2HPO_4 (71643, Sigma-Aldrich), 5 mM KH_2PO_4, pH 6.5), when about 1/3rd of the bacterial lawn was cleared and developed on NN agar (1.5% agar in 8.8 mM KH_2PO_4 and 3.4 mM Na_2HPO_4) at 1.7 × 10^6 cells/cm^2 until the desired developmental stage was reached.

Promoter-*lacZ* constructs

To prepare promoter-*lacZ* fusions of the putative *Pvio* prespore genes g4562 (*psp1*), g2696 (*psp2*) and g2380 (*psp3*), their respectively 2.1, 1.2 and 2.2 kb 5′intergenic regions were

amplified from *Pvio QSvi11* genomic DNA using Phusion high-fidelity DNA polymerase (F530S, ThermoFisher Scientific) and the oligonucleotide primer pairs g4562pF/g4562pR, g2696pF/g2696pR or g2380pF/g2380pR (Table 1) which harbour XbaI and BglII sites in the forward (F) and reverse (R) primers respectively. After XbaI/BglII digestion, the amplified products were cloned into XbaI/BglII digested vector pDdgal-17 (Harwood & Drury, 1990), which fuses the start codon and usually the first few amino-acids of the inserted gene in frame with *LacZ*. The constructs were validated by DNA sequencing using primers Gal17F and Gal17R (Table 1) and transformed into *Pvio QSvi11* as described by (Narita *et al.*, 2020), except that initial overnight starvation was at 5×10^6 cells/ml instead of 5×10^5.

Transformants were initially selected at 50 µg/ml G418 (G4181, Formedium) on 1/3rd SM agar with G418 resistant *Escherichia coli* (Narita *et al.*, 2020), which was raised to 100 µg/ml G418 for *psp1-lacZ* and *psp2-lacZ* transformed cells and to 200 µg G418/ml for *psp3-lacZ*, which was expressed relatively weakly. The generated plasmids are deposited in the *Dictyostelium* Stock Center http://dictybase.org/StockCenter/StockCenter.html.

β-galactosidase histochemistry

Cells transformed with *lacZ* constructs were washed free from bacteria and 20 µl aliquots of 10^8 cells/ml were plated on 2×2 cm squares of nitrocellulose filters (GE10600002, Merck) supported by NN agar. Filters with developing structures were transferred to squares of Whatman 3MM paper (WHA3030931, Merck) soaked in 0.5% glutaraldehyde (354400, Sigma-Aldrich) in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 (71469, Sigma-Aldrich), 10 mM KCl (P3911, Sigma-Aldrich) and 1 mM $MgSO_4$, pH 7.0) and incubated for 5 min. Structures were next fully submersed in 0.25% glutaraldehyde and 1% Tween-20 (P2287, Sigma-Aldrich) in Z buffer for 10 min and then washed and stained with X-gal (XGAL001, Formedium) as described previously (Dingermann *et al.*, 1989).

Prespore gene induction and β-galactosidase assay

For time courses, *Pvio* cells transformed with *psp1-LacZ* or *psp2-LacZ* were developed on nitrocellulose filters as above, harvested from filters by mixing on a Vortex Genie 2 (Z258423, Sigma-Aldrich) in 0.5 ml 1x Z-buffer, frozen at -20°C and lysed by freeze-thawing three times, with thawing under vigorous shaking. β-galactosidase activity was assayed in 100 µl lysate and protein in 20 µl lysate in triplicate.

For gene induction cells were starved for 4 h on NN agar, aggregates were gently dissociated and cells resuspended to 2×10^6 cells/ml. 50 µl of cell suspension was incubated in triplicate with 50 µl of variables or water in microtiter plates for 6 h at 22°C, while shaking on an IKA MTS4 microplate shaker (0003208002 IKA, Oxford, UK). Cells were frozen at -20°C and lysed as above.

To assay β-galactosidase activity, lysed cells were supplemented with 10 µl 40 mM chlorophenolred-β-D-galactopyranoside (CPRG)(59767 Sigma-Aldrich) and for the gene induction experiments with 30 µl 2.5x Z-buffer (1% mercapto-ethanol (M3148, Sigma-Aldrich) in 150 mM Na_2HPO_4, 100 mM NaH_2PO_4, 25 mM KCl, 7.5 mM $MgSO_4$, pH 7.0). The optical density at 574 nm was measured at regular intervals using a Multiskan SkyHigh Microplate Spectrophotometer (A51119500C, Thermo Fisher Scientific). The β-galactosidase activity (ΔOD_{574}/min) was calculated from one or more intervals where OD_{574} increased linearly and standardized on the protein content of the lysates as assayed with Bradford reagent (5000205 Bio-rad).

Results

Identification of *Pvio* prespore genes

D. discoideum prespore genes were originally identified as genes that were highly enriched in the prespore fraction of dissociated slugs, separated on density gradients (Mehdy *et al.*, 1983; Ratner & Borth, 1983). Many of these genes were found to encode spore coat proteins, which accumulate as an inner lining of Golgi-derived prespore vesicles in the posterior of early slugs (Fosnaugh & Loomis, 1989). In the final phase of fruiting body formation, the vesicles fuse with the plasma membrane and their inner lining forms the first layer of the spore wall. Another prespore gene, *pspA*, encodes a cell surface protein, recognized by monoclonal antibody MUD1 (Early *et al.*, 1988). The expression of all these genes starts shortly after aggregation and is induced and maintained by micromolar

Table 1. Oligonucleotide primers.

Primer ID	Sequence	gene name
g4562pF	GGG**TCTAGA**GGGTAGGAGAGGTAACAAATG	*psp1*
g4562pR	GGG**AGATCT**TGACTTCATTTTATTCTTATTAATTTAATTC	
g2696pF	CCC**TCTAGA**CATATGATAGGTGTATGCAGATTTAAG	*psp2*
g2696pR	CCC**AGATCT**CATTGTACTAGTTATATTTTAAATTATTTTAGTAATGTTAC	
g2380pF	GTGTCAGACATTTTATGAATATATATAGTTA**TCTAGA**AAG	*psp3*
g2380pR	CC**AGATCT**TGGCTTCATTTATTTATGTATATGTG	
Gal17F	TATGGTAAAACTTGAATTGATCCTCTAG	
Gal17R	CATCCTGCAGTAAGCTTGGTACCGAGATC	

concentrations of cAMP (Mehdy & Firtel, 1985; Schaap & Van Driel, 1985). The genes encoding the vesicle-associated proteins also require intracellular cAMP acting on PKA for expression (Hopper *et al.*, 1993).

The spore coat proteins are members of a large family that share FOLN (Follistatin-N-terminal domain-like, EGF-like) domains and often Dicty_spore_N domains. The family shows gene gain and loss in the different dictyostelid taxon groups, precluding unequivocal assessment of orthology. While many *Ddis* members have known functions in spore coat assembly (Srinivasan *et al.*, 1999; West, 2003), functions in the stalk or cyst wall for others cannot be excluded. To identify *Pvio* prespore genes, I therefore sought for the closest relatives of the well-characterized *Ddis* spore coat genes *cotA*, *cotB*, *cotC*, *cotD*, *cotE*, *pspA*, *pspB* and *pspD* (Fosnaugh & Loomis, 1989; Grant *et al.*, 1985; Metcalf *et al.*, 2007; Smith *et al.*, 1989; Takemoto *et al.*, 1990; Yoder *et al.*, 1994) and *SP45,* a prespore gene of the group 2 species *Ppal* (Gregg & Cox, 2000). To assist subdivision in orthologous groups, a second group 4 species, *D. purpureum* (*Dpur*), and the group 3 species *D. lacteum* (*Dlac*) were also included in the search. A phylogeny was inferred from the closest BLASTp hits to the 7 *Ddis* genes, which was annotated with protein functional domains and developmental and cell-type specific expression profiles of the genes (Figure 1). *PspA* was not detected in the *Pvio* genome. The relatives of *cotB*, *cotA*, *cotD* and *pspB* separate into clades with single members in each investigated species. Across species, these genes and most of the others increase expression in aggregates, but down-regulate expression in mature fruiting bodies. While in *Ddis* slugs, their expression is strongly prespore-enriched, later in mature fruiting bodies, expression is higher in cup and stalk cells than in spores. Note that the developmental- and cell-type specificity profiles are obtained from separate RNAseq experiments, which means that expression data between profiles cannot be compared in an absolute sense and expression in all mature cell types may be relatively low. *cotE* is expressed late in development and is directly transported to the spore coat (Metcalf *et al.*, 2007). A previously identified *Pvio* spore gene *Pvio_g1612* (Narita *et al.*, 2020) belongs to a similar class of late spore genes. From the six detected *Pvio* prespore gene candidates, I selected three genes *Pvio*_g4562 (*psp1*), *Pvio*_g2696 (*psp2*) and *Pvio*_g2380 (*psp3*) with relatively high expression levels and for which the full 5'intergenic sequence was present in the (draft) genome.

Expression pattern of putative *Pvio* prespore genes

The 5'intergenic regions of the three candidate prespore genes were fused to the *lacZ* reporter gene and *Pvio* cells, transformed with either construct, were developed on non-nutrient agar and stained with X-gal (Figure 2A). The promoter activity patterns of the three genes were very similar. β-galactosidase activity was first observed inside newly

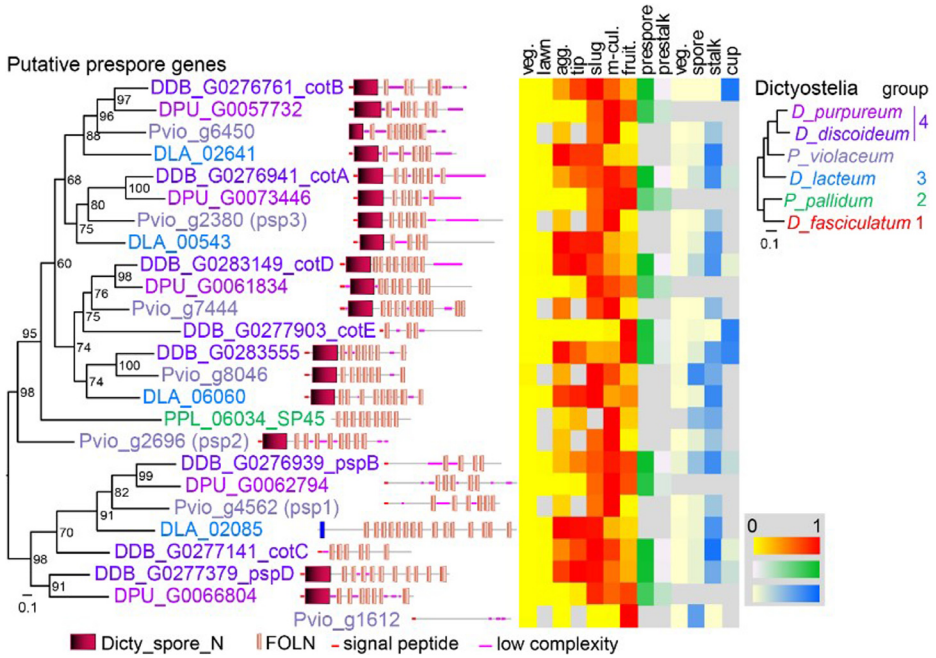

Figure 1. Search for *Pvio* prespore genes. The *Pvio* (Narita *et al.*, 2020), *D. lacteum* (Glöckner *et al.*, 2016) and *D.purpureum* (Sucgang *et al.*, 2011) proteomes were queried for relatives of 7 well-documented *D. discoideum* and one *P. pallidum* prespore genes using BLASTp. The top hits were aligned using ClustalOmega (Sievers & Higgins, 2014) and a phylogenetic tree was inferred with IQtree (Nguyen *et al.*, 2015). The tree is annotated with the functional domains of the proteins, as analysed by SMART (Schultz *et al.*, 1998), and with relative expression levels of the genes during the *Ddis* developmental cycle and in its purified cell types, as retrieved from published RNAseq experiments (Glöckner *et al.*, 2016; Kawabe *et al.*, 2023; Kin *et al.*, 2018; Narita *et al.*, 2020; Parikh *et al.*, 2010). A multigene phylogeny of taxon-group representative Dictyostelia (Schilde *et al.*, 2019) is shown in top right corner.

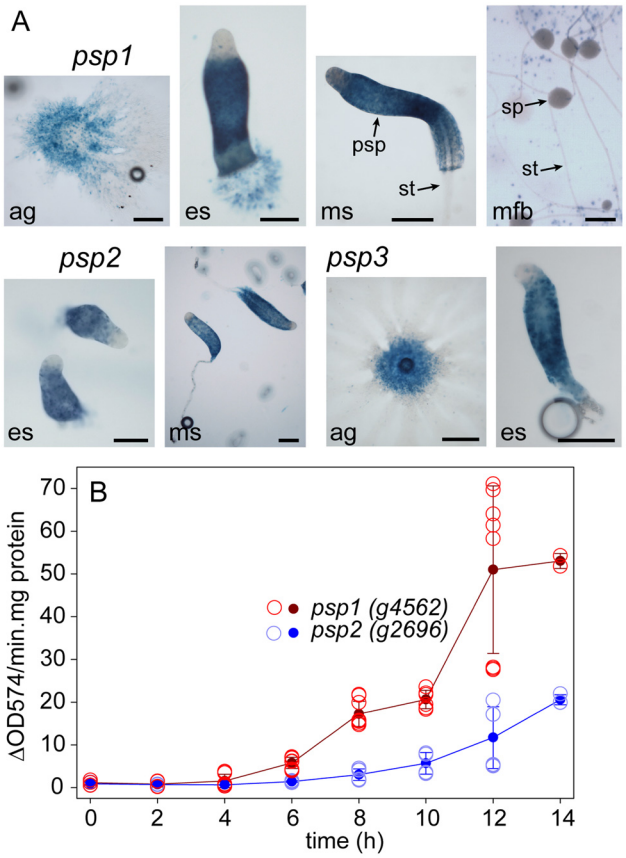

Figure 2. Pattern and timing of *Pvio* prespore gene expression. A. *Pattern. Pvio* cells transformed with *lacZ* fusions with the 5'intergenic regions of the *Pvio psp1* (*g4562*), *psp2* (*g2696*) and *psp3* (*g2380*) genes were developed on nitrocellulose filters supported by NN agar. Developing structures were fixed and stained with X-gal from 4 h and imaged under transmitted light. Bars: 200 μm. ag: aggregates, es and ms: early and mid sorogens, mfb: mature fruiting bodies, psp, sp and st: prespore, spore and stalk cells. **B**. *Developmental timing.* $4x10^6$ cells developed on 2x2 cm^2 filters were harvested and frozen at the indicated time periods of starvation, and then lysed and assayed spectrophotometrically for β-galactosidase activity as described in Methods. Two time courses with triplicate filters were examined for the *psp1-LacZ* cells and one time course for the *psp2-LacZ* cells. The open circles represent the activity standardized on protein of the individual filters and the closed circles and error bars the mean and SD of all measurements. The underlying data are archived at https://osf.io/bawv6/ PvioPresporeGeneInduction.xlsx, sheet Fig.2BData. (Schaap, 2024).

formed aggregates with little to no activity in the inflowing streams and then partitioned to the rear 4/5th of the emerging sorogen (es). The tip region, which was initially free of β-galactosidase activity, became proportionally smaller as the sorogen matured and the newly formed stalks contained few or no stained cells. While in freshly formed fruiting bodies the spore heads were deeply stained, β-galactosidase activity disappeared as the spores matured further.

Overall the expression pattern of the *Pvio* prespore genes was similar to that of the *Ddis* prespore genes *pspA* and *cotA,B* and *C,* except the anterior prespore free region remains considerably larger in *Ddis* throughout development. Compared to *Ddis* sorogens, the signature prespore vesicles are also present much closer to the tip in *Pvio* sorogens, where they undergo degradation as the cells are differentiating into stalk cells (Schaap *et al.*, 1985).

Genes *psp1*, *psp2* and *psp3* are therefore reliable markers of prespore differentiation.

To estimate the developmental stage when cells were competent for gene induction by signal molecules, *psp1-lacZ* and *psp2-lacZ* cells, developed on filters, were also assayed for β-galactosidase activity by a quantitative spectrophotometric assay. Figure 2B showed that activity started to increase at 4 h of starvation (when cells had formed streaming aggregates) up to the last time point at 14 h (when cells were in the mid-sorogen stage). *Psp1-lacZ* cells showed about 2.5 fold higher activity as *psp2-lacZ* cells.

Effects of *Ddis* and *Pvio* signal molecules on *Pvio* prespore gene expression

Prespore gene expression in *Ddis* is induced by cAMP acting on cARs and further promoted by GABA (gamma-aminobutyric acid), an unknown steroid and the peptides SDF1 and SDF2. The latter four signals eventually activate PKA, which is also directly activatable by the membrane-permeant cAMP analogue 8Br-cAMP (Anjard *et al.*, 1998; Anjard & Loomis, 2006; Anjard & Loomis, 2008; Anjard *et al.*, 2009; Wang *et al.*, 1999). c-di-GMP activates PKA in prestalk cells to induce stalk

cell maturation (Chen & Schaap, 2012; Chen *et al.*, 2017), while DIF-1 induces basal disc differentiation in *Ddis* (Saito *et al.*, 2008). cAMP and glorin are the chemoattractants that mediate *Ddis* and *Pvio* aggregation respectively, while folate mediates attraction to the bacterial food source (Konijn *et al.*, 1967; Pan *et al.*, 1972; Shimomura *et al.*, 1982). cGMP is a second messenger mediating chemotaxis (Mato *et al.*, 1977). Except for SDF1 and SDF2 which are not commercially available, I compared the effect of all these compounds on the induction of *Pvio psp1-lacZ* and *psp2-lacZ* expression in 4 h starved cells shaken in buffer for 6 h with cells developing on solid substratum for the same period. The compounds were added in the concentration range where they are active in *Ddis*. These are 10^{-5} to 10^{-3} M for cAMP (Schaap & Van Driel, 1985), $>10^{-10}$ M for DIF-1 (Masento *et al.*, 1988), 10^{-7} to 10^{-5} M for c-di-GMP (Chen & Schaap, 2012), 10^{-9} to 10^{-4} M for GABA (Anjard & Loomis, 2006), 10 to 15 mM for 8Br-cAMP (Yamada *et al.*, 2018). Glorin, folate and cGMP were used in the range where cAMP induces prespore gene expression.

Figure 3 shows that without added compounds *psp1-lacZ* activity increased about 4-fold over 6 h incubation, while *psp2-lacZ* activity increased about 6-fold (compare black diamonds to coloured circles at 0 M). None of the added compounds had any significant effect on this increase over their entire concentration range. Note that the control (water) values fluctuate a bit around 1 for the individual concentration series, which likely reflects accumulated pipetting errors, since this value was set at 1 for the averaged control values of all series. The fluctuations from 1 when variables were added is mostly not greater than that at the control, indicating that they represent the pipetting error. On the other hand, the cells developed for 6 h on solid substratum (to mid-sorogens) showed 5 to 60-fold higher activity for *psp1* and *psp2*, respectively, than those incubated in dilute suspensions.

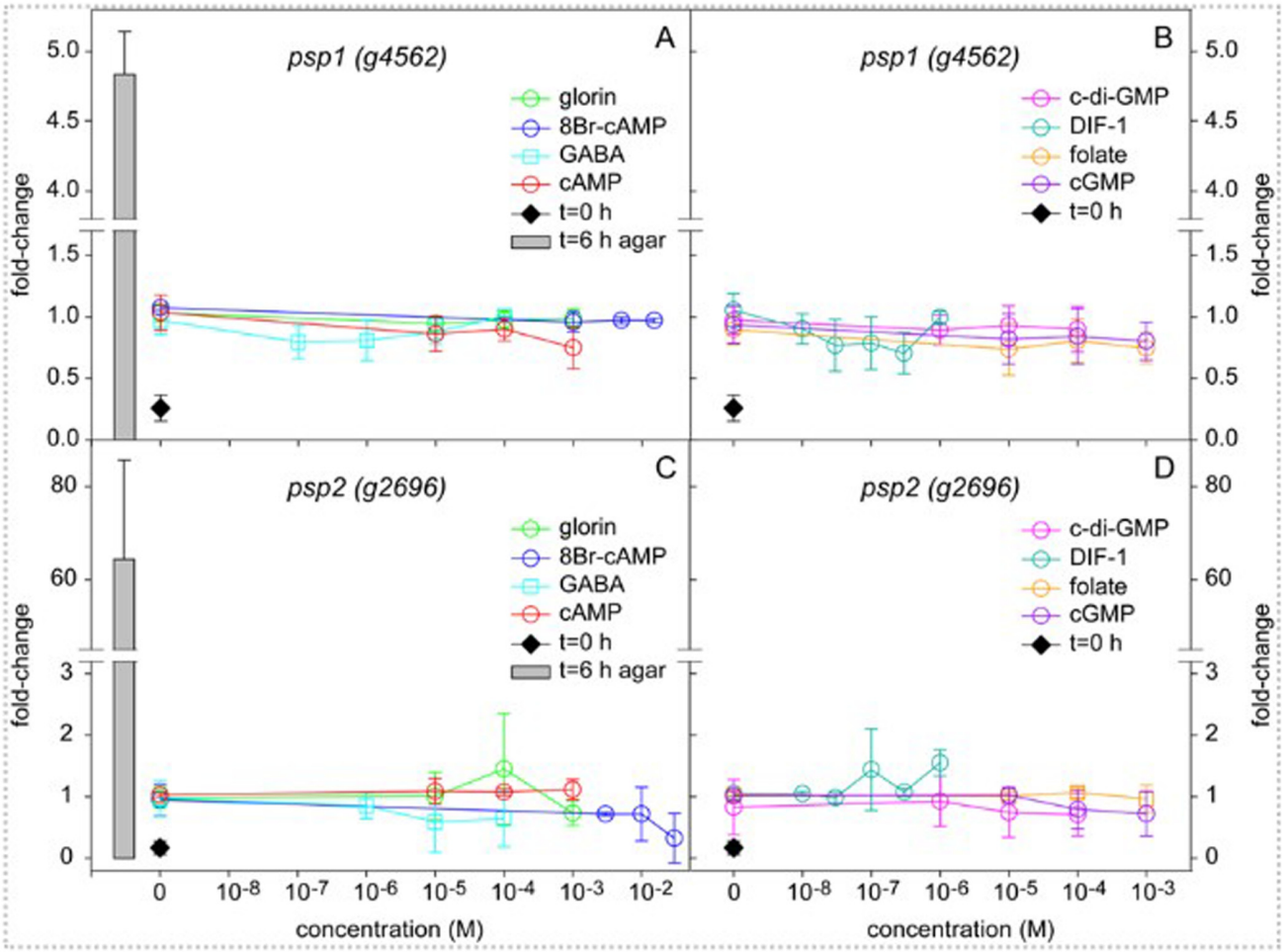

Figure 3. Induction of prespore gene expression by signal molecules. *Pvio psp1-lacZ* and *psp2-lacZ* cells were starved for 4 h on NN agar, harvested and incubated at 10^6 cells/ml in PB for 6 h with the indicated concentrations of signal molecules or developed on as 10^6 cells per 1 cm^2 filter on NN agar. At 0 and 6 h of incubation cells were frozen, lysed and assayed for β-galactosidase activity and protein content. The results are expressed as fold-change of the ΔOD_{574}/min.mg protein values over the averaged control (water) values for all concentration series at t=6 h. Data represent means and SD of 2 or 3 independent experiments performed in triplicate for each variable. The underlying data are archived at https://osf.io/baww6/ PvioPresporeGeneInduction.xlsx, sheet Fig.3Data (Schaap, 2024).

Evidently, the cells received some signal during normal development, that is different from any of the signals that were added during incubation in suspension, although a small amount of an endogenous inducer was also produced by the suspended cells.

Discussion

Three marker genes for prespore differentiation were identified in *Pvio*. Their promoters were fused to *LacZ* and *a* convenient bioassay for detection of prespore-inducing signals was developed from *Pvio* cells expressing these constructs. Unlike *Ddis* in group 4 (Schaap & Van Driel, 1985) and *Ppal* in group 2 (Kawabe *et al.*, 2009) who use secreted cAMP acting on cARs to induce prespore genes, *Pvio,* a sister species to group 4 did not require its supposedly single cAR for (pre)spore differentiation (Kawabe & Schaap, 2023). Using the novel constructs, it was evident that *Pvio* prespore genes are not inducible by cAMP (Figure 3A,C), which indicates that normal development of the *Pvio cAR* knock-out was not due to an undiscovered cAR taking over its function.

The *Pvio* chemoattractant glorin could also not induce expression of prespore genes and neither did folate, a chemoattractant used for food seeking in group 4 (Pan *et al.*, 1972) and for aggregation in the group 3 species *D. minutum* (De Wit & Konijn, 1983). Dissimilar to *Ddis*, the *Pvio* prespore-inducing signal is unlikely to be a chemoattractant. cGMP, an intermediate of cAMP- and folate-induced chemotaxis (Mato *et al.*, 1977) had also no effect on prespore gene induction. Competent *Pvio* cells showed a low level of prespore gene induction in suspension, but this endogenous induction was neither stimulated nor inhibited by the *Ddis* signals c-di-GMP and DIF-1 (Figure 3B,D) which induce stalk and basal disc expression, respectively (Chen & Schaap, 2012; Saito *et al.*, 2008), and in case of DIF-1 inhibit prespore differentiation (Wang *et al.*, 1987).

Many signals act late in *Ddis* development to regulate the timely maturation of spore and stalk cells by controlling the levels of intracellular cAMP and thereby the activation of PKA. These signals such as GABA, SDF1 and SDF2 and an unidentified steroid act in synergy with cAR activation by extracellular cAMP to induce maximal expression of prespore genes and exocytosis of prespore vesicles, which are lined with the first layer of the spore coat. The effects of these late signals can be mimicked by the membrane-permeant PKA agonist 8Br-cAMP (Anjard *et al.*, 1998; Anjard & Loomis, 2006; Anjard & Loomis, 2008; Anjard *et al.*, 2009; Wang *et al.*, 1999). A secreted glycoprotein, PsiA, promotes prespore differentiation in the presence of cAMP in an unknown manner (Kawata *et al.*, 2004). *psiA* is member of a clade of 15 PA14 genes, which are amplified only in group 4, and it therefore has no ortholog in *Pvio*.

Neither GABA nor up to 30 mM of 8Br-cAMP stimulated *Pvio* prespore gene expression. *Pvio* cells can take up to 8Br-cAMP, since earlier experiments showed that 10 mM 8Br-cAMP could restore aggregation and spore differentiation in *Pvio* lacking the two adenylate cyclases *acaA* and *acrA*. In *Ddis*, *AcrA* has a specific role in synthesizing cAMP for PKA activation in spore maturation and the late functions of *AcrA* and PKA were apparently conserved in *Pvio* (Kawabe & Schaap, 2023). Only the early role of secreted cAMP in prespore induction is not conserved and the question arises what the missing signal might be.

Dictyostelia contain many species- or group-specific genes involved secondary metabolism such as polyketide synthases and terpene synthases (Chen *et al.*, 2018; Heidel *et al.*, 2011; Zucko *et al.*, 2007). Several secondary metabolites were previously identified that act on cell differentiation, such as DIF-1 (Morris *et al.*, 1987), MPBD (Narita *et al.*, 2011) and dictyodene D (Günther *et al.*, 2022). In addition, dictyostelid genomes contain large and highly variable families of cell surface or secreted glycoproteins, such as the IPT/TIG and PA40 domain families with known members like tgrB1/C1 and psiA being involved in developmental transitions (Hirose *et al.*, 2015; Kawata *et al.*, 2004). In the assay used here cells still clumped together during incubation, making it unlikely that direct cell-cell contact was the missing signal. While it will be a daunting task to identify any candidate as the *Pvio* prespore inducer, the availibitily of a sensitive bio-assay based on the *psp1_LacZ* transformed *Pvio* cells is a step towards its completion.

The loss of secreted cAMP function in *Pvio* multicellular development does not only concern prespore induction but also the regulation of multicellular morphogenesis, which is in groups 4 (Singer *et al.*, 2019), 3 (Alvarez-Curto *et al.*, 2005; Schaap *et al.*, 1984) and 2 (Kawabe *et al.*, 2009; Kawabe *et al.*, 2012) mediated by cAMP as chemoattractant. With the associated proteins involved in the timely synthesis and processing of the novel signal(s), this is from an evolutionary perspective an immense lineage-specific innovation in a pathway essential for species survival. It emphasizes that evolution does not always involve small stepwise improvements, but can proceed in remarkably large spurts.

Ethics and consent

Ethical approval and consent were not required.

Data availibility

Underlying data

OSF: Prespore gene induction in Polysphondylium violaceum. DOI 10.17605/OSF.IO/BAWV6

This project contains the following underlying data:

- A spreadsheet entitled PvioPresporeGenInduction.xlsx

This project is under license by *CC0 1.0 Universal*

The generated plasmids are deposited in the *Dictyostelium* Stock Center http://dictybase.org/StockCenter/StockCenter.html.

Author contributions

P.S. designed the study, performed the experiments and wrote the manuscript.

References

Alvarez-Curto E, Rozen DE, Ritchie AV, *et al.*: **Evolutionary origin of cAMP-based chemoattraction in the social amoebae.** *Proc Natl Acad Sci U S A.* 2005; **102**(18): 6385–6390.
PubMed Abstract | Publisher Full Text | Free Full Text

Anjard C, Chang WT, Gross J, *et al.*: **Production and activity of Spore Differentiation Factors (SDFs) in *Dictyostelium*.** *Development.* 1998; **125**(20): 4067–4075.
PubMed Abstract | Publisher Full Text

Anjard C, Loomis WF: **GABA induces terminal differentiation of *Dictyostelium* through a $GABA_B$ receptor.** *Development.* 2006; **133**(11): 2253–2261.
PubMed Abstract | Publisher Full Text

Anjard C, Loomis WF: **Cytokinins induce sporulation in *Dictyostelium*.** *Development.* 2008; **135**(5): 819–827.
PubMed Abstract | Publisher Full Text

Anjard C, Su Y, Loomis WF: **Steroids initiate a signaling cascade that triggers rapid sporulation in *Dictyostelium*.** *Development.* 2009; **136**(5): 803–812.
PubMed Abstract | Publisher Full Text | Free Full Text

Chen X, Köllner TG, Shaulsky G, *et al.*: **Diversity and functional evolution of terpene synthases in dictyostelid social amoebae.** *Sci Rep.* 2018; **8**(1): 14361.
PubMed Abstract | Publisher Full Text | Free Full Text

Chen ZH, Schaap P: **The prokaryote messenger c-di-GMP triggers stalk cell differentiation in *Dictyostelium*.** *Nature.* 2012; **488**(7413): 680–683.
PubMed Abstract | Publisher Full Text | Free Full Text

Chen ZH, Singh R, Cole C, *et al.*: **Adenylate Cyclase A acting on PKA mediates induction of stalk formation by Cyclic diguanylate at the *Dictyostelium* organizer.** *Proc Natl Acad Sci U S A.* 2017; **114**(3): 516–521.
PubMed Abstract | Publisher Full Text | Free Full Text

de Wit RJ, Konijn TM: **Identification of the acrasin of *Dictyostelium minutum* as a derivative of folic acid.** *Cell Differ.* 1983; **12**(4): 205–210.
PubMed Abstract | Publisher Full Text

Dingermann T, Reindl N, Werner H, *et al.*: **Optimization and in situ detection of *Escherichia coli* β-galactosidase gene expression in *Dictyostelium Discoideum*.** *Gene.* 1989; **85**(2): 353–362.
PubMed Abstract | Publisher Full Text

Du Q, Schilde C, Birgersson E, *et al.*: **The cyclic AMP phosphodiesterase RegA critically regulates encystation in social and pathogenic amoebas.** *Cell Signal.* 2014; **26**(2): 453–459.
PubMed Abstract | Publisher Full Text | Free Full Text

Early AE, Williams JG, Meyer HE, *et al.*: **Structural characterization of *Dictyostelium Discoideum* prespore-specific gene D19 and of its product, cell surface glycoprotein PsA.** *Mol Cell Biol.* 1988; **8**(8): 3458–3466.
PubMed Abstract | Publisher Full Text | Free Full Text

Fosnaugh KL, Loomis WF: **Spore coat genes SP60 and SP70 of *Dictyostelium Discoideum*.** *Mol Cell Biol.* 1989; **9**(11): 5215–5218.
PubMed Abstract | Publisher Full Text | Free Full Text

Funamoto S, Anjard C, Nellen W, *et al.*: **cAMP-dependent Protein Kinase regulates *Polysphondylium pallidum* development.** *Differentiation.* 2003; **71**(1): 51–61.
PubMed Abstract | Publisher Full Text

Glöckner G, Lawal HM, Felder M, *et al.*: **The multicellularity genes of dictyostelid social amoebas.** *Nat Commun.* 2016; **7**: 12085.
PubMed Abstract | Publisher Full Text | Free Full Text

Grant WN, Welker DL, Williams KL: **A polymorphic, prespore-specific cell surface glycoprotein is present in the extracellular matrix of *Dictyostelium Discoideum*.** *Mol Cell Biol.* 1985; **5**(10): 2559–2566.
PubMed Abstract | Publisher Full Text | Free Full Text

Gregg KY, Cox EC: **Spatial and temporal expression of a *Polysphondylium* spore-specific gene.** *Dev Biol.* 2000; **224**(1): 81–95.
PubMed Abstract | Publisher Full Text

Günther M, Reimer C, Herbst R, *et al.*: **Yellow polyketide pigment suppresses premature hatching in social amoeba.** *Proc Natl Acad Sci U S A.* 2022; **119**(43): e2116122119.
PubMed Abstract | Publisher Full Text | Free Full Text

Harwood AJ, Drury L: **New vectors for expression of the *E.coli lacZ* gene in *Dictyostelium*.** *Nucleic Acids Res.* 1990; **18**(14): 4292.
PubMed Abstract | Publisher Full Text | Free Full Text

Harwood AJ, Hopper NA, Simon MN, *et al.*: **Culmination in *Dictyostelium* is regulated by the cAMP-dependent Protein Kinase.** *Cell.* 1992; **69**(4): 615–624.
PubMed Abstract | Publisher Full Text

Heidel AJ, Lawal HM, Felder M, *et al.*: **Phylogeny-wide analysis of social amoeba genomes highlights ancient origins for complex intercellular communication.** *Genome Res.* 2011; **21**(11): 1882–1891.
PubMed Abstract | Publisher Full Text | Free Full Text

Hirose S, Santhanam B, Katoh-Kurosawa M, *et al.*: **Allorecognition, via TgrB1 and TgrC1, mediates the transition from unicellularity to multicellularity in the social amoeba *Dictyostelium Discoideum*.** *Development.* 2015; **142**(20): 3561–3570.
PubMed Abstract | Publisher Full Text | Free Full Text

Hopper NA, Harwood AJ, Bouzid S, *et al.*: **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.* 1993; **12**(6): 2459–2466.
PubMed Abstract | Publisher Full Text | Free Full Text

Kalla SE, Queller DC, Lasagni A, *et al.*: **Kin discrimination and possible cryptic species in the social amoeba *Polysphondylium violaceum*.** *BMC Evol Biol.* 2011; **11**: 31.
PubMed Abstract | Publisher Full Text | Free Full Text

Kawabe Y, Du Q, Narita TB, *et al.*: **Emerging roles for diguanylate cyclase during the evolution of soma in dictyostelia.** *BMC Ecol Evol.* 2023; **23**(1): 60.
PubMed Abstract | Publisher Full Text | Free Full Text

Kawabe Y, Morio T, James JL, *et al.*: **Activated cAMP receptors switch encystation into sporulation.** *Proc Natl Acad Sci U S A.* 2009; **106**(17): 7089–7094.
PubMed Abstract | Publisher Full Text | Free Full Text

Kawabe Y, Schaap P: **Development of the dictyostelid *Polysphondylium violaceum* does not require secreted cAMP.** *Biol Open.* 2023; **12**(2): bio059728.
PubMed Abstract | Publisher Full Text | Free Full Text

Kawabe Y, Schilde C, Du Q, *et al.*: **A conserved signalling pathway for amoebozoan encystation that was co-opted for multicellular development.** *Sci Rep.* 2015; **5**: 9644.
PubMed Abstract | Publisher Full Text | Free Full Text

Kawabe Y, Weening KE, Marquay-Markiewicz J, *et al.*: **Evolution of self-organisation in Dictyostelia by adaptation of a non-selective phosphodiesterase and a matrix component for regulated cAMP degradation.** *Development.* 2012; **139**(7): 1336–1345.
PubMed Abstract | Publisher Full Text | Free Full Text

Kawata T, Nakagawa M, Shimada N, *et al.*: **A gene encoding, prespore-cell-inducing factor in *Dictyostelium Discoideum*.** *Dev Growth Differ.* 2004; **46**(4): 383–392.
PubMed Abstract | Publisher Full Text

Kin K, Forbes G, Cassidy A, *et al.*: **Cell-type specific RNA-seq reveals novel roles and regulatory programs for terminally differentiated *Dictyostelium* cells.** *BMC Genomics.* 2018; **19**(1): 764.
PubMed Abstract | Publisher Full Text | Free Full Text

Konijn TM, Van De Meene JG, Bonner JT, *et al.*: **The acrasin activity of adenosine-3′,5′-cyclic phosphate.** *Proc Natl Acad Sci U S A.* 1967; **58**(3): 1152–1154.
PubMed Abstract | Publisher Full Text | Free Full Text

Mann SKO, Richardson DL, Lee S, *et al.*: **Expression of cAMP-dependent protein kinase in prespore cells is sufficient to induce spore cell differentiation in *Dictyostelium*.** *Proc Natl Acad Sci U S A.* 1994; **91**(22): 10561–10565.
PubMed Abstract | Publisher Full Text | Free Full Text

Masento MS, Morris HR, Taylor GW, *et al.*: **Differentiation-inducing factor from the slime mould *Dictyostelium discoideum* and its analogues. Synthesis, structure and biological activity.** *Biochem J.* 1988; **256**(1): 23–28.
PubMed Abstract | Publisher Full Text | Free Full Text

Mato JM, Krens FA, Van Haastert JPM, *et al.*: **3′: 5′-cyclic AMP dependent 3′: 5′-cyclic GMP accumulation in *Dictyostelium discoideum*.** *Proc Natl Acad Sci U S A.* 1977; **74**(6): 2348–2351.
PubMed Abstract | Publisher Full Text | Free Full Text

Mehdy MC, Firtel RA: **A secreted factor and cyclic AMP jointly regulate cell-type-specific gene expression in *Dictyostelium discoideum*.** *Mol Cell Biol.* 1985; **5**(4): 705–713.
PubMed Abstract | Publisher Full Text | Free Full Text

Mehdy MC, Ratner D, Firtel RA: **Induction and modulation of cell-type-specific gene expression in Dictyostelium.** *Cell.* 1983; **32**(3): 763–771.
PubMed Abstract | Publisher Full Text

Metcalf T, van der Wel H, Escalante R, *et al.*: **Role of SP65 in assembly of the *Dictyostelium discoideum* spore coat.** *Eukaryot Cell.* 2007; **6**(7): 1137–1149.
PubMed Abstract | Publisher Full Text | Free Full Text

Morris HR, Taylor GW, Masento MS, *et al.*: **Chemical structure of the morphogen differentiation inducing factor from *Dictyostelium discoideum*.** *Nature.* 1987; **328**(6133): 811–814.
PubMed Abstract | Publisher Full Text

Narita TB, Kawabe Y, Kin K, *et al.*: **Loss of the polyketide synthase StlB results in stalk cell overproduction in *Polysphondylium violaceum*.** *Genome Biol Evol.* 2020; **12**(5): 674–683.
PubMed Abstract | Publisher Full Text | Free Full Text

Narita TB, Koide K, Morita N, *et al.*: ***Dictyostelium* hybrid polyketide synthase, SteelyA, produces 4-methyl-5-pentylbenzene-1,3-diol and induces spore maturation.** *FEMS Microbiol Lett.* 2011; **319**(1): 82–87.
PubMed Abstract | Publisher Full Text

Nguyen LT, Schmidt HA, von Haeseler A, *et al.*: **IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies.** *Mol Biol Evol.* 2015; **32**(1): 268–274.
PubMed Abstract | Publisher Full Text | Free Full Text

Pan P, Hall EM, Bonner JT: **Folic acid as second chemotactic substance in the cellular slime moulds.** *Nat New Biol.* 1972; **237**(75): 181–182.
PubMed Abstract | Publisher Full Text

Parikh A, Miranda ER, Katoh-Kurasawa M, *et al.*: **Conserved developmental**

transcriptomes in evolutionarily divergent species. *Genome Biol.* 2010; **11**(3): R35.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Ratner D, Borth W: **Comparison of diffentiating *Dictyostelium discoideum* cell types separated by an improved method of density gradient centrifugation.** *Exp Cell Res.* 1983; **143**(1): 1–13.
PubMed Abstract | **Publisher Full Text**

Ritchie AV, van Es S, Fouquet C, *et al.*: **From drought sensing to developmental control: evolution of cyclic AMP signaling in social amoebas.** *Mol Biol Evol.* 2008; **25**(10): 2109–2118.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Romeralo M, Skiba A, Gonzalez-Voyer A, *et al.*: **Analysis of phenotypic evolution in Dictyostelia highlights developmental plasticity as a likely consequence of colonial multicellularity.** *Proc Biol Sci.* 2013; **280**(1764): 20130976.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Saito T, Kato A, Kay RR: **DIF-1 induces the basal disc of the *Dictyostelium* fruiting body.** *Dev Biol.* 2008; **317**(2): 444–453.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Schaap P: **Evolution of developmental signalling in Dictyostelid social amoebas.** *Curr Opin Genet Dev.* 2016; **39**: 29–34.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Schaap P: **PvioPresporeGeneInduction.xlsx.** *Open Science Framework.* 2024.
http://www.doi.org/10.17605/OSF.IO/BAWV6

Schaap P, Konijn TM, Van Haastert PJ: **cAMP pulses coordinate morphogenetic movement during fruiting body formation of *Dictyostelium minutum.*** *Proc Natl Acad Sci U S A.* 1984; **81**(7): 2122–2126.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Schaap P, Pinas JE, Wang M: **Patterns of cell differentiation in several cellular slime mold species.** *Dev Biol.* 1985; **111**(1): 51–61.
Publisher Full Text

Schaap P, Van Driel R: **Induction of post-aggregative differentiation in *Dictyostelium discoideum* by cAMP. Evidence of involvement of the cell surface cAMP receptor.** *Exp Cell Res.* 1985; **159**(2): 388–398.
PubMed Abstract | **Publisher Full Text**

Schaap P, Winckler T, Nelson M, *et al.*: **Molecular phylogeny and evolution of morphology in the social amoebas.** *Science.* 2006; **314**(5799): 661–663.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Schilde C, Lawal HM, Kin K, *et al.*: **A well supported multi gene phylogeny of 52 dictyostelia.** *Mol Phylogenet Evol.* 2019; **134**: 66–73.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Schilde C, Skiba A, Schaap P: **Evolutionary reconstruction of pattern formation in 98 *Dictyostelium* species reveals that cell-type specialization by lateral inhibition is a derived trait.** *EvoDevo.* 2014; **5**: 34.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Schultz J, Milpetz F, Bork P, *et al.*: **SMART, a simple modular architecture research tool: identification of signaling domains.** *Proc Natl Acad Sci U S A.* 1998; **95**(11): 5857–5864.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Shimomura O, Suthers HLB, Bonner JT: **Chemical identity of the acrasin of the cellular slime mold *Polysphondylium violaceum.*** *Proc Natl Acad Sci U S A.* 1982; **79**(23): 7376–7379.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Sievers F, Higgins DG: **Clustal omega, accurate alignment of very large numbers of sequences.** *Methods Mol Biol.* 2014; **1079**: 105–116.
PubMed Abstract | **Publisher Full Text**

Singer G, Araki T, Weijer CJ: **Oscillatory cAMP cell-cell signalling persists during multicellular *Dictyostelium* development.** *Commun Biol.* 2019; **2**: 139.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Smith E, Gooley AA, Hudson GC, *et al.*: **Glycoproteins that exhibit extensive size polymorphisms in *Dictyostelium discoideum*.** *Genetics.* 1989; **122**(1): 59–64.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Srinivasan S, Alexander H, Alexander S: **The prespore vesicles of *Dictyostelium discoideum*. Purification, characterization, and developmental regulation.** *J Biol Chem.* 1999; **274**(50): 35823–35831.
PubMed Abstract | **Publisher Full Text**

Sucgang R, Kuo A, Tian X, *et al.*: **Comparative genomics of the social amoebae *Dictyostelium discoideum* and *Dictyostelium purpureum.*** *Genome Biol.* 2011; **12**(2): R20.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Takemoto K, Takeuchi I, Tasaka M: **cAMP regulation of the expression of prespore-specific genes, SP96 and Dp87, in disaggregated slug cells of *Dictyostelium discoideum*.** *Cell Differ Dev.* 1990; **31**(2): 89–96.
PubMed Abstract | **Publisher Full Text**

Wang N, Soderbom F, Anjard C, *et al.*: **SDF-2 induction of terminal differentiation in *Dictyostelium discoideum* is mediated by the membrane-spanning sensor kinase DhkA.** *Mol Cell Biol.* 1999; **19**(7): 4750–4756.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Wang M, Van Driel R, Schaap P: **Cyclic AMP-phosphodiesterase induces dedifferentiation of prespore cells in *Dictyostelium discoideum* slugs: evidence that cyclic AMP is the morphogenetic signal for prespore differentiation.** *Development.* 1988; **103**(3): 611–618.
Publisher Full Text

Wang M, Van Haastert PJM, Schaap P: **Multiple effects of differentiation-inducing factor on prespore differentiation and cyclic-AMP signal transduction in *Dictyostelium*.** *Differentiation.* 1987; **33**(3): 24–28.
Publisher Full Text

West CM: **Comparative analysis of spore coat formation, structure, and function in *Dictyostelium*.** *Int Rev Cytol.* 2003; **222**: 237–293.
PubMed Abstract | **Publisher Full Text**

Yamada Y, Cassidy A, Schaap P: **The transcription factor Spores Absent A is a PKA dependent inducer of *Dictyostelium* sporulation.** *Sci Rep.* 2018; **8**(1): 6643.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Yoder BK, Mao J, Erdos GW, *et al.*: **Identification of a new spore coat protein gene in the cellular slime mold *Dictyostelium discoideum*.** *Dev Biol.* 1994; **163**(1): 49–65.
PubMed Abstract | **Publisher Full Text**

Zucko J, Skunca N, Curk T, *et al.*: **Polyketide synthase genes and the natural products potential of *Dictyostelium discoideum*.** *Bioinformatics.* 2007; **23**(19): 2543–2549.
PubMed Abstract | **Publisher Full Text**

Open Peer Review

Current Peer Review Status: ✔ ✔ ✔

Version 2

Reviewer Report 14 December 2024

https://doi.org/10.21956/openreseurope.20582.r48164

© **2024 Morimoto Y.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

✔ **Yusuke V. Morimoto**
Kyushu Institute of Technology, Fukuoka, Japan

The author has made sufficient additions and corrections and this manuscript should be index as a final version.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular genetics, Cell biology, Biophysics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 18 November 2024

https://doi.org/10.21956/openreseurope.19845.r45744

© **2024 Saran S.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

✔ **Shweta Saran**
Jawaharlal Nehru University, New Delhi, Delhi, India

The article entitled “Novel invention of spore induction in a sister species to group 4 Dictyostelia” by Pauline Schaap is accepted.

The main findings in this article are the following:
- She identified the *Polyspondylium violaceum (Pvio)* genes g4562 (psp1), g2696 (psp2) and g2380 (psp3) which were homologs of *Dictyostelium discoideum* spore coat genes and studied their expression patterns.
- cAMP had no effect on prespore gene induction and neither had the *Pvio* chemoattractant glorin nor the *Dictyostelium discoideum* chemoattractants and differentiation inducers folate, c-di-GMP, DIF-1, GABA, cGMP and 8Br-cAMP.

Thus, she concluded that *Pvio* lineage uniquely evolved a novel genetic network for synthesis, detection and processing of the unknown signal in a timely manner that triggers its main survival strategy.

The manuscript is well written, experiments are neatly designed and figures are clear. The discussion is very well written. There are earlier paper (Hanna *et al*., Expt Cell Res, 1979, 122:265-271; Shimomura *et al*., 1982, PNAS 79: 7376-7379) where they have talked about morphogenesis and stalk and spore formation that is independent of cAMP (the known chemoattractant in *D. discoideum*, belonging to the same group IV). In her earlier paper, (Kawabe and Schaap, Biol Open, 2023, 12), they have shown that in *Pvio* secreted cAMP was not required for development but intracellular cAMP was required for the initiation of development and spore maturation. This study was an outcome of many deletion mutants generated that were components of cAMP signalling pathway. Here an elegant method was employed to study the different spore genes.
I was just wondering if any metabolites could be involved?

References

1. Kawabe Y, Schaap P: Development of the dictyostelid Polysphondylium violaceum does not require secreted cAMP.*Biol Open*. 2023; **12** (2). PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: cell and developmental biology working with Dictyostelium

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 23 Nov 2024

Pauline Schaap

I thank reviewer 3 for her concise description and positive opinion of the manuscript. With regard to her question whether any metabolites could be involved, I assume that she meant metabolites of the compounds that were tested. This is possible, but one then would assume at least some effect of the added variables as they would likely be metabolized during the 6 h incubation period.

Competing Interests: No competing interests were disclosed.

Reviewer Report 14 November 2024

https://doi.org/10.21956/openreseurope.19845.r45746

© 2024 Morimoto Y. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

?

Yusuke V. Morimoto
Kyushu Institute of Technology, Fukuoka, Japan

This study suggests the existence of an unknown signaling pathway in cellular slime molds, a finding that is of great evolutionary interest both in the study of cellular slime molds and in the study of eukaryotic signal transduction more broadly. The draft is carefully verified in comparison with previous findings. However, some of the descriptions are inadequate and need to be revised.

1. In the results of the *lacZ* reporter, data corresponding to the description are not adequately presented.“β-galactosidase activity was first observed in the centre of streaming aggregates”
This is shown by the photo of the ag of psp3 in Fig 2A? However, the same activity is not seen in the ag of psp1.
“the newly formed stalks contained few or no stained cells.”
Do you have any pictures showing this?
“While in freshly formed fruiting bodies the spore heads were deeply stained, β-galactosidase activity
disappeared as the spores matured further.”
Arrows are needed to indicate where to look in Fig 2A. Need descriptions such as which parts are the spores.

2. Fig2B and Fig3 do not show about *psp3*. Can you explain why only *psp1* and *psp2* were used?

3. Since it is mentioned that “The compounds were added in the concentration range where they

are active in *Ddis*.", references are needed for each active concentration of each compounds.

4. I could not find the numbers in the following text from the Figure 3. "Figure 3 shows that without added compounds psp1-lacZ activity increased about 4-fold over 6 h incubation, while psp2-lacZ activity increased about 6-fold."
Please provide a detailed explanation or modify the figure to make them easier to read.
And, it should be shown that there is no change in the water-only control.

5. The activity values for *psp1* and *psp2* in Fig3 are reversed from those in Fig2. A description of what causes this difference is needed.

6. Page6, Line13: "stalk cels" should be "stalk cells".

7. Page7, Line2 "psp1-lacZ" and "psp1-lacZ" should be in italic.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular genetics, Cell biology, Biophysics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Nov 2024

Pauline Schaap

I am grateful to the reviewer for his in-depth review. Revisions were made to the manuscript to address his comments as outlined below:

1. In the results of the lacZ reporter, data corresponding to the description are not adequately presented."β-galactosidase activity was first observed in the centre of streaming aggregates" This is shown by the photo of the ag of psp3 in Fig 2A? However, the same activity is not seen in the ag of psp1.
Au: *I agree that the description allowed multiple interpretations. I meant that it was observed in the actual aggregate and not the inflowing streams. The sentence was altered into:"β-galactosidase activity was first observed inside newly formed aggregates with little to no activity in the inflowing streams".*

"the newly formed stalks contained few or no stained cells." Do you have any pictures showing this? *Au: this was evident in figure 2A right top panels, but is due to its low resolution not clear in the published PDF version. I have replaced the third top panel with a figure at higher magnification.* "While in freshly formed fruiting bodies the spore heads were deeply stained, β-galactosidase activity disappeared as the spores matured further." Arrows are needed to indicate where to look in Fig 2A. Need descriptions such as which parts are the spores.
Au: *Arrows and descriptions are added to figure 2A*

2. Fig2B and Fig3 do not show about psp3. Can you explain why only psp1 and psp2 were used?
Au*: The psp3-LacZ plasmid took much longer to construct than the other two and was only finished after the induction experiments were mostly done. The psp3 promoter activity was also very weak and we therefore did not consider it necessary to repeat all experiments again with this promoter.*

3. Since it is mentioned that "The compounds were added in the concentration range where they are active in Ddis.", references are needed for each active concentration of each compounds.
Au*: This sentence is now followed up by a referenced listing of active concentration ranges*

4. I could not find the numbers in the following text from the Figure 3. "Figure 3 shows that without added compounds psp1-lacZ activity increased about 4-fold over 6 h incubation, while psp2-lacZ activity increased about 6-fold." Please provide a detailed explanation or modify the figure to make them easier to read.
Au*: a similar comment (#2) was also made by reviewer 1. I added the line "(compare black diamonds to coloured circles at 0 M)" to the text to clarify this. The black diamonds are controls taken at t=0 h and the coloured circles at this position are water controls taken at t=6h. Note that the underlying experimental data can be accessed in a spreadsheet using hyperlink https://osf.io/bawv6/, as indicated in the legend to figure 3* And, it should be shown that there is no change in the water-only control. *Au: the change between 0 and 6 h at 0 M are the roughly 4 and 6-fold changes in basal activity*

5. The activity values for psp1 and psp2 in Fig3 are reversed from those in Fig2. A description of what causes this difference is needed.
Au*: This seems particularly the case for cells developed on agar. In figure 2B the β-galactosidase activity is expressed as an absolute value (ΔOD574/min.mg protein), while in figure 3 the activity*

is expressed as fold-change over the water control value to highlight the extent of gene induction (or lack thereof) by the different variables. The high value for psp2 agar control simply reflects that compared to psp1 its water control value (in the denominator) is very low, thus producing a high fold-change.

6. Page6, Line13: “stalk cels” should be “stalk cells”.
Au: *corrected*

7. Page7, Line2 “psp1-lacZ” and “psp1-lacZ” should be in italic
Au: *corrected*

Competing Interests: No competing interests were disclosed.

Reviewer Report 13 November 2024

https://doi.org/10.21956/openreseurope.19845.r45740

© **2024 Saito T.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Tamao Saito
Sophia University, Tokyo, Japan

Molecular phylogenetic analysis by the author has divided cellular slime mould into four groups. The most well-studied D. discoideum (Ddis) belongs to the most evolved Group 4 (G4).
Previous studies have suggested that there is a significant evolutionary gap between G4 and G3. Therefore, the analysis of morphological formation using P. violaceum (Pvio), a sister clade of G4, is important in tracing the path by which G4 evolved from G3.
Finding differentiation markers is therefore a fundamental and essential step. The author identified spore markers based on meticulous validation and showed the pattern formation and the timing of marker gene expression. In addition, bio-assay have been established that use these markers to find signaling molecules. The bioassay method is particularly useful for Pvio, as it is conceivable that some signaling molecules are the same as those of Ddis but work through different pathways.
In this study, a major step in the evolution between G4 and G3 was demonstrated.

I only have a few suggestions and question. None of these are major.

Page 6 fifth line from bottom of left column. Shouldn't “we” be changed to “I”?

Page 7 last sentence; In Figure 3, it was not clear where the activity of each gene was shown to increase by 4- to 6-fold without the added compounds.

I would like to comment on the signaling molecules involved in the expression of the prespore

genes. Among the secondary metabolites listed in the Discussion, MPBD and Dictyoquinone have been shown to be involved in cAMP signaling in Ddis and also in spore maturation. In this context, it would be appropriate to investigate their effects in this study.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biochemistry, Chemical ecology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 23 Nov 2024

Pauline Schaap

I thank the reviewer for her insightful review. I made minor revisions to the manuscript and respond to her numbered comments below:

1. Page 6 fifth line from bottom of left column. Shouldn't “we” be changed to “I”?

Au: This and three more instances of “we” were replaced by “I”

1. Page 7 last sentence; In Figure 3, it was not clear where the activity of each gene was shown to increase by 4- to 6-fold without the added compounds.

Au: This is evident by comparing the black diamonds (t=0 h) with the coloured circles (t=6 h) at 0 M of additives. I have added a note in the text to clarify this.

1. I would like to comment on the signaling molecules involved in the expression of the prespore genes. Among the secondary metabolites listed in the Discussion, MPBD and Dictyoquinone have been shown to be involved in cAMP signaling in Ddis and also in spore maturation. In this context, it would be appropriate to investigate their effects in this study.

Au: MPBD was shown to promote spore maturation by indirectly activating PKA. We here

tested other compounds (GABA and 8Br-cAMP) that promote spore maturation by activating PKA. They had no effect on prespore gene induction, so we did not consider it necessary to test more compounds in this category. Dictyoquinone is to our knowledge not commercially available.

Competing Interests: No competing interests were disclosed.