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RESEARCH ARTICLE

Development of the dictyostelid *Polysphondylium violaceum* does not require secreted cAMP

Yoshinori Kawabe and Pauline Schaap*

ABSTRACT

Group 4 Dictyostelia, like *Dictyostelium discoideum*, self-organize into aggregates and fruiting bodies using propagating waves of the chemoattractant cAMP, which are produced by a network containing the adenylate cyclase AcaA, cAMP receptors (Cars) and the extracellular cAMP phosphodiesterase PdsA. Additionally, AcaA and the adenylate cyclases AcrA and AcgA produce secreted cAMP for induction of aggregative and prespore gene expression and intracellular cAMP for PKA activation, with PKA triggering initiation of development and spore and stalk maturation. Non-group 4 species also use secreted cAMP to coordinate post-aggregative morphogenesis and prespore induction but use other attractants to aggregate. To understand how cAMP's role in aggregation evolved, we deleted the *acaA*, *carA* and *pdsA* genes of *Polysphondylium violaceum*, a sister species to group 4. *acaA*$^-$ fruiting bodies had thinner stalks but otherwise developed normally. Deletion of *acrA*, which was similarly expressed as *acaA*, reduced aggregation centre initiation and, as also occurred after *D. discoideum acrA* deletion, caused spore instability. Double *acaA*$^-$*acrA*$^-$ mutants failed to form stable aggregates, a defect that was overcome by exposure to the PKA agonist 8Br-cAMP, and therefore likely due to reduced intracellular cAMP. The *carA*$^-$ and *pdsA*$^-$ mutants showed normal aggregation and fruiting body development. Together, the data showed that *P. violaceum* development does not critically require secreted cAMP, while roles of intracellular cAMP in initiation of development and spore maturation are conserved. Apparently, cell-cell communication underwent major taxon-group specific innovation in Dictyostelia.

KEY WORDS: Evolution of cell-cell communication, Clade-specific invention, Cell-type specialization, CAMP oscillations, Morphogenetic movement, Chemotaxis

INTRODUCTION

Multicellularity evolved independently in seven of the eight major divisions of eukaryotes (Brown et al., 2012; Du et al., 2015; Tice et al., 2016) and in prokaryotes (Lyons and Kolter, 2015). Multicellular forms range from simple clumps or mats of cells to a myriad of complex organisms composed of many specialized cells, arranged in tissues and organs. The regulation of cell-type specialization and the proper positioning of the specialized cells within the organism requires extensive cell to cell communication mechanisms.

Comparative genomics has highlighted that many proteins involved in mediating and processing intercellular communication are deeply conserved throughout multicellular lineages (Gold et al., 2019; Srivastava et al., 2010), while those involved in intracellular processing can often be retraced to their unicellular ancestors (King et al., 2008; Suga et al., 2013). Nevertheless, there are only few narratives that connect signalling mechanisms in a unicellular ancestor with developmental control in a multicellular descendant. One example is the co-option of a transcription factor, RSL1, that mediates stress response in the unicellular alga *Chlamydomonas reinhardtii* into a developmental role as RegA, inducing somatic cell fate in its multicellular relative *Volvox carteri* (König and Nedelcu, 2020). Another example is the evolution of cell-type specialization and morphogenesis in the Dictyostelia from an ancestral stress response.

Dictyostelia are members of Amoebozoa, a eukaryote division that mostly consists of single-celled amoebas, which encapsulate to form walled cysts when starved. Instead, starving Dictyostelia aggregate to form multicellular fruiting bodies that consists of walled spores and stalk cells. The model species *D. discoideum* (*Ddis*), uses cAMP as attractant to coordinate aggregation and fruiting body morphogenesis. For this role, cAMP is secreted in pulses, which are generated by a network that contains the adenylate cyclase AcaA (Pitt et al., 1992), G-protein coupled cAMP receptors (Cars) (Sun et al., 1990), an extracellular phosphodiesterase, PdsA (Sucgang et al., 1997), and some intracellular components. Secreted cAMP also regulates developmental gene expression and induces the expression of prespore genes. In addition, cAMP acts an intracellular messenger for external signals that trigger the growth to development transition and the encapsulation of spores and stalk cells. Here, cAMP activates cAMP-dependent protein kinase (PKA) and is mostly synthesized by the adenylate cyclases AcgA and AcrA and hydrolysed by the intracellular phosphodiesterase RegA (Du et al., 2015; Loomis, 2014).

Molecular phylogeny partitions Dictyostelia into four major and some minor groups (Schaap et al., 2006; Schilde et al., 2019). Many species in groups 1, 2 and 3 can still individually encyst in addition to forming fruiting bodies, but group 4, which contains *Ddis*, has lost encystation (Romeralo et al., 2013). Comparative studies revealed that the roles of PKA, AcgA, AcrA and RegA in *Ddis* spore and stalk encapsulation are evolutionary derived from roles as intermediates for stress-induced encystation in both Dictyostelia and single-celled Amoebozoa (Du et al., 2014; Kawabe et al., 2015; Ritchie et al., 2008).

Group 4 species all use cAMP as the attractant for aggregation, but most species in groups 1, 2 and 3 appear to use the dipeptide glorin (Romeralo et al., 2013), that was initially identified as the attractant of *Polysphondylium violaceum* (*Pvio*) (Shimomura et al., 1982). Deletion of *pdsA* and the *carA* and *carB* genes of the group 2

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species *Polysphondylium pallidum* (*Ppal*) did not affect aggregation (as expected) but disrupted fruiting body morphogenesis. The *carA*$^-$*carB*$^-$ cells also lost prespore differentiation and differentiated as cysts in the aberrant fruiting structures (Kawabe et al., 2009, 2012). These data indicated that secreted cAMP coordinated post-aggregative morphogenesis and induced prespore differentiation in *Ppal*, as it does in *Ddis*, and supported a hypothesis whereby first intracellular and next extracellular cAMP signalling in Dictyostelia evolved from cAMP-mediated encystation in the unicellular ancestor (Kin and Schaap, 2021).

We recently developed gene knock-out procedures and generated genome and transcriptome sequence data for *Pvio*, a species that resides in a small sister group to group 4 (Narita et al., 2020). This position and the fact that it only has single copies of the *acaA*, *car* and *pdsA* genes makes *Pvio* uniquely suited to investigate how secreted cAMP signalling evolved to its very dominant role in group 4. The gene disruption studies led to the surprising finding that in *Pvio* development extracellular cAMP signalling plays no role of significance at all.

RESULTS

Investigation of AcaA function in *P. violaceum*

Pvio is one of the earliest identified species of Dictyostelia (Brefeld, 1884) and is characterized by aggregates with inflowing streams, the formation of whorls of side branches from cell masses that pinch off from the primary sorogen and the violet colour of its mature spores. It is the founding species of the genus *Polysphondylium*, which all share the whorled fruiting bodies, but not the violet colour. Classification based on molecular data later showed that the whorled phenotype was polyphyletic (Schaap et al., 2006; Schilde et al., 2019) and that white polyphondylids were members of group 2 of the 4 major dictyostelid taxon groups, while *Pvio* was part of a small sister clade to group 4, with the latter containing *Ddis* and other species that use cAMP as attractant. The *Pvio* attractant was identified as a dipeptide of glutamate and ornithine, called glorin (Shimomura et al., 1982) and appears to be widely used among non-group 4 species (Romeralo et al., 2013). Genome and transcriptome data for *Pvio* Qsvi11 are available (Narita et al., 2020) and this strain can be transformed with vectors harbouring G418 or hygromycin selection cassettes. Gene knock-out by homologous transformation proved thus far to be very efficient, usually occurring in 80-100% of transformed clones (Narita et al., 2020). The whorled phenotype of Qsvi11 is, however, variable and whorls are often sparse, making it difficult to evaluate effects of gene manipulation on whorl formation.

AcaA is encoded by a single copy gene in *Pvio* (Fig. S1) and to assess its role in post-aggregative morphogenesis, we deleted *acaA* by homologous recombination (Fig. S2A). The *acaA*$^-$ clones aggregated normally and formed fruiting bodies with somewhat thinner and longer stalks than those of wild-type cells (Fig. 1A). This was also evident in Calcofluor stained structures at higher magnification, where the length/width ratio of *acaA*$^-$ stalk cells was greater than that of wild-type stalk cells (Fig. 1B). The *acaA*$^-$ mutant formed normal elliptical spores.

Phenotypes of *Pvio acrA*$^-$ and *acaA*$^-$*acrA*$^-$ double mutants

Similar to other Dictyostelia, *Pvio* has two other adenylate cyclase genes, *acrA* and *acgA* (Fig. S1). *Ddis acrA*$^-$ cells form fruiting bodies with thin stalks and spores that germinate precociously in the spore head (Soderbom et al., 1999), while *acgA*$^-$ mutants show only sporulation defects (Alvarez-Curto et al., 2007; Van Es et al., 1996). *Pvio acgA* is predominantly expressed in spores in late development (Fig. S1), while *acrA* is like *acaA* upregulated during aggregation and stalk-enriched (Fig. S1). To examine the expression pattern of both genes in developing structures, we transformed wild-type *Pvio* with fusion constructs of the *acaA* or *acrA* promoters and the *lacZ* reporter. Expression of β-galactosidase from either promoter was enriched in aggregation centres and was later present throughout the primary and secondary sorogens and the stalk (Fig. S3). Expression from the *acaA* promoter was somewhat stronger at the sorogen tips. Because the expression pattern of *acrA* is almost the same as that of *acaA*, we investigated whether AcrA might compensate for loss of AcaA in *Pvio* by disrupting *acrA* in wild-type and *acaA*$^-$ cells (Fig. S2B).

The *Pvio acrA* knock-out clones formed large aggregates and robust fruiting bodies (Fig. 2A). Measurement of aggregate density showed that *acrA*$^-$ cells initiated about eight times less aggregates/mm^2 than wild-type cells (Fig. 2B), which is the likely cause of the increased size of their aggregates and fruiting bodies. Aggregation of *acrA*$^-$ cells also seemed a bit delayed, but because the timing of aggregation is also variable in wild-type *Pvio*, we cannot state this with certainty. Calcofluor staining revealed that *acrA*$^-$ cells initially formed normal spores and stalk cells in early fruiting bodies (Fig. 2C, 12 h), but that 4 h later the spore head contained many amoebas and empty spore walls, indicating that spores had precociously germinated. Precocious spore germination is also reported for *Ddis acrA*$^-$ (Soderbom et al., 1999). Expression of *acrA* in *acrA*$^-$ cells restored both the reduced aggregate density (Fig. 2A) and precocious germination defects of *acrA*$^-$ (Fig. 2C),

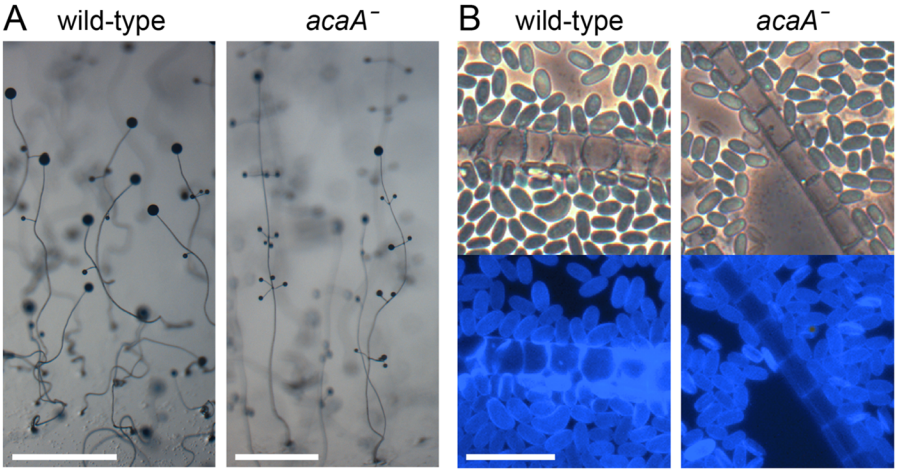

Fig. 1. Development and differentiation of the *Pvio acaA*$^-$ mutants. *Pvio* wild-type and *acaA*$^-$ cells were incubated overnight at 4°C on KK2 agar at 10^6 cells/cm^2 and then at 22°C until mature fruiting bodies had formed. (A) Fruiting body morphology of wild type and *acaA*$^-$ cells. Scale bars: 1.0 mm. (B) Mature fruiting structures were stained with 0.001% Calcofluor *in situ* and photographed under phase contrast (top) and epifluorescence (bottom). Scale bar: 20 µm.

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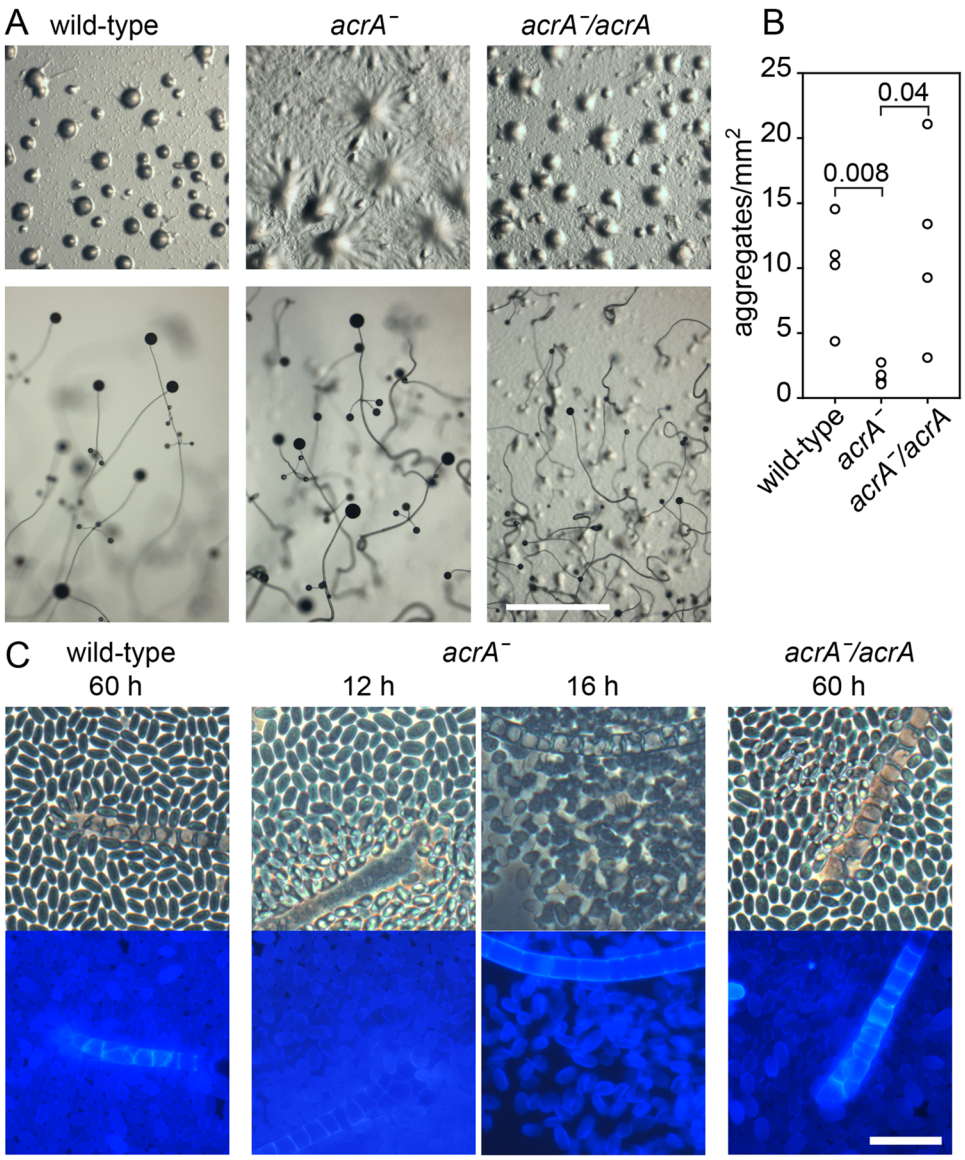

Fig. 2. Phenotype of *Pvio acrA*⁻ and *acrA*⁻/*AcrA* mutants. (A) Development and fruiting body morphology. *Pvio* wild-type, *acrA*⁻ and *acrA*⁻ cells, complemented with *acrA* expressed from its own promoter, were plated on KK2 agar at 10^6 cells/cm^2 and incubated until cells had formed aggregates (top) and mature fruiting bodies (bottom). Scale bar: 1 mm. (B) Aggregate density. After aggregation was completed, the number of aggregates per mm^2 was determined. Data of four independent experiments, measuring 108 to 322 aggregates for per cell line, each, are shown with *P*-values of a *t*-test. The data passed normality and equal variance tests. (C) Sporulation. Cells were starved overnight at 4°C and then for 12 h to 60 h at 22°C. Mature fruiting structures were stained with 0.001% Calcofluor *in situ* and photographed under phase contrast (top) and epifluorescence (bottom). Scale bar: 20 µm.

indicating that these phenotypes were caused by *acrA* gene disruption.

The *Pvio acaA*⁻*acrA*⁻ mutant was severely defective. It initially aggregated upon starvation but never formed sorogens (Fig. 3). Instead, the aggregated cells dispersed within a few hours and then formed aggregates again. This process was repeated several times at about 5-7 h intervals during at least 24 h. Normal development was restored by expression of either *acaA* or *acrA* from their own promoters (Fig. 4). Each of the complemented strains formed fruiting bodies, but the spores of *acaA*⁻*acrA*⁻ transformed with *acaA* germinated in spore head (Fig. 4B), as was the case for *acrA*⁻ mutants. The restoration of fruiting body formation indicates that *acaA* and *acrA* have overlapping roles in stabilizing aggregates to initiate progression into fruiting body formation.

Restoration of *acaA*⁻*acrA*⁻ development by 8Br-cAMP

In *Ddis*, fruiting body formation requires both extracellular cAMP acting on Cars for organisation of morphogenesis and induction of prespore gene expression (Singer et al., 2019; Wang et al., 1988) and intracellular cAMP acting on PKA for induction of spore and stalk cell maturation (Harwood et al., 1992; Hopper et al., 1993). To address which cAMP signalling defect caused the *acaA*⁻/*acrA*⁻ phenotype, *Pvio acaA*⁻/*acrA*⁻ cells were developed on agar containing 8Br-cAMP, a membrane-permeant cAMP analogue with high affinity for PKA, but not Cars (Van Haastert and Kien, 1983). At 2.5 mM 8Br-cAMP, stable tipped aggregates were formed that did not develop further, but at 5 mM and more so at 10 mM 8Br-cAMP, the tipped mounds developed into stalked structures with spore heads, which, though morphologically aberrant, showed normal elliptical spores and vacuolated stalk cells (Fig. 5). These results show that direct activation of PKA compensates fully for defective aggregation, sporulation and stalk cell differentiation in the *acaA*⁻*acrA*⁻ and partially for defective morphogenesis.

Effects of deletion of *Pvio carA* and *PdsA* and Sp-cAMPS

The partial restoration of *Pvio acaA*⁻*acrA*⁻ development by 8Br-cAMP indicates that AcaA and AcrA have a shared role in providing intracellular cAMP for PKA activation. However, this does not exclude that secreted cAMP pulses are not important for *Pvio* development as well. In *Ddis*, both the cAMP receptor CarA and the extracellular cAMP phosphodiesterase PdsA are essential for generating cAMP pulses, while CarA also mediates cAMP induction of chemotaxis and

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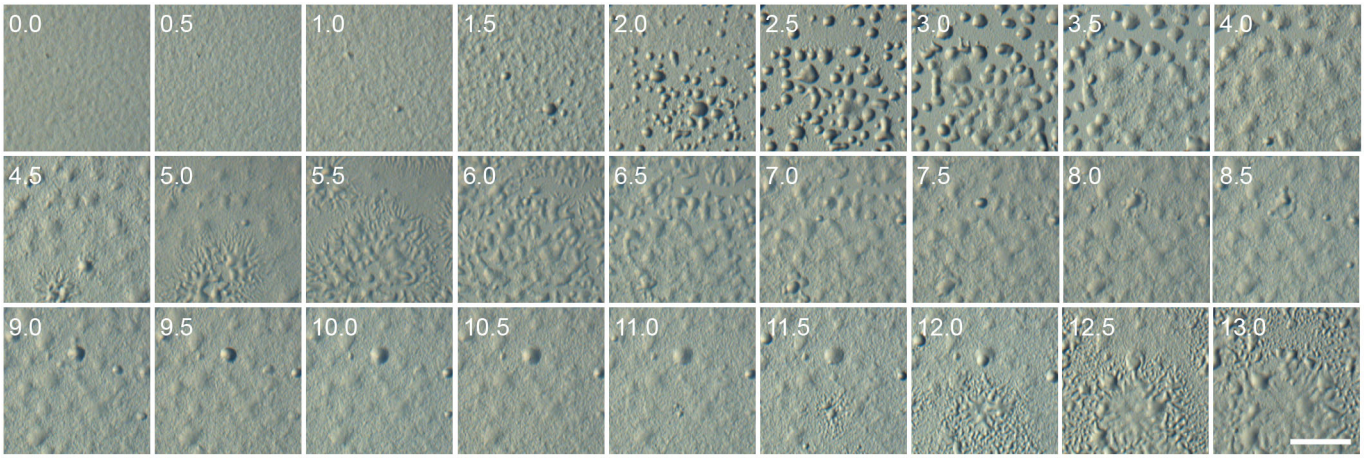

Fig. 3. Development of a *Pvio acaA⁻acrA⁻* mutant. *Pvio acaA⁻acrA⁻* cells were incubated overnight at 4°C on KK2 agar at 10^6 cells/cm^2 and then transferred to 22°C. The numbers on the upper left indicate hours of incubation at 22°C. Scale bar: 0.5 mm.

prespore gene expression (Schaap and Van Driel, 1985; Sucgang et al., 1997; Sun et al., 1990). BlastP searches revealed that *Pvio* has only a single *pdsA* and a single *carA* gene, which were respectively amplified once and three times in group 4 (Fig. S4).

We deleted the single *carA* and *pdsA* genes to evaluate roles for secreted cAMP in *Pvio* (Fig. S5). Both the *carA⁻* and *pdsA⁻* mutants aggregated normally and constructed fruiting bodies (Fig. 6A). Calcofluor staining revealed that these fruiting bodies were composed of mature spores and stalk cells whose morphologies were also normal. These results suggest that secreted cAMP does not play a significant role in *Pvio* aggregation, fruiting body development or cell differentiation. To further test involvement of oscillatory cAMP signalling in *Pvio*, we developed cells on agar containing 10, 50 or 100 µM on the slowly hydrolysable cAMP analogue Sp-cAMPS, which inhibits cAMP responses like chemotaxis and cAMP relay that are subject to adaptation, and thereby inhibit *Ddis* development, starting from 0.5 µM (Rossier et al., 1978). Fig. S6 shows that up to 100 µM Sp-cAMPS had no effect on *Pvio* aggregation and development into fruiting bodies, consolidating the evidence that *Pvio* does not require dynamic extracellular cAMP signalling.

DISCUSSION

AcaA does not critically regulate *P. violaceum* development

Phylogenetic comparative methods showed that major innovations occurred in the last common ancestor (LCA) to the group 4 dictyostelids, such as the formation of robust solitary unbranched fruiting bodies with two novel somatic cell types, the early fate mapping of spore and stalk cells and extensive slug migration. Species in groups 1, 2 and 3 form smaller grouped or branched fruiting bodies with stalk cells as the only somatic cell type, and the stalk is formed by local redifferentiation of prespore cells. The changes in group 4 are accompanied by the loss of encystation as an alternative survival strategy and the use of secreted cAMP pulses to coordinate chemotactic aggregation (Romeralo et al., 2013; Schilde et al., 2014).

Deletion of *car* and *pdsA* genes in the group 2 species *Ppal* indicated that non-group 4 species still used secreted cAMP to organize post-aggregative morphogenesis and prespore differentiation (Alvarez-Curto et al., 2005; Kawabe et al., 2002, 2009, 2012). This notion was supported by findings that the group 3 species *D. minutum*, displayed cAMP stimulated cAMP synthesis (Schaap, 1985) and optical density waves after aggregation, with the latter being disrupted by the non-hydrolysable cAMP analogue Sp-cAMPS (Schaap et al., 1984). Sp-cAMPS also disrupted post-aggregative morphogenesis of most other non-group 4 species (Romeralo et al., 2013).

With its position in the closest sister clade to group 4, *Pvio* is well placed for investigating the molecular changes that occurred in the LCA to group 4 that caused the dramatic innovations in this group. An added advantage is the efficiency of gene knock-out in *Pvio* (Narita et al., 2020) and that its attractant, glorin, is known (Shimomura et al., 1982). To particularly understand the evolution of secreted cAMP signalling, we deleted the single gene encoding

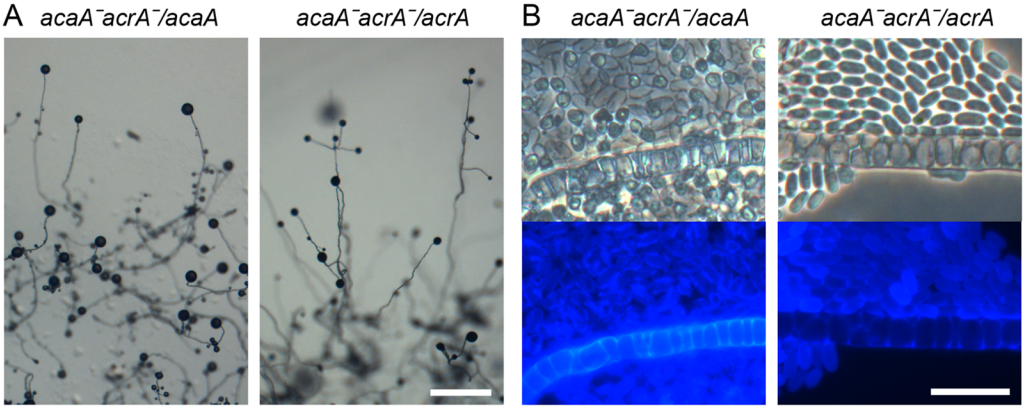

Fig. 4. Complementation of *acaA⁻acrA⁻* with *acaA* or *acrA*. (A) Fruiting body morphology. The *acaA⁻acrA⁻* mutant was transformed with *acaA* or *acrA* expressed from their own promoter. The transformants were developed into fruiting bodies and photographed. Scale bar: 1 mm. (B) Spore and stalk cells. Mature fruiting structures were stained with 0.001% Calcofluor *in situ* and photographed under phase contrast (top) and epifluorescence (bottom). Scale bar: 20 µm.

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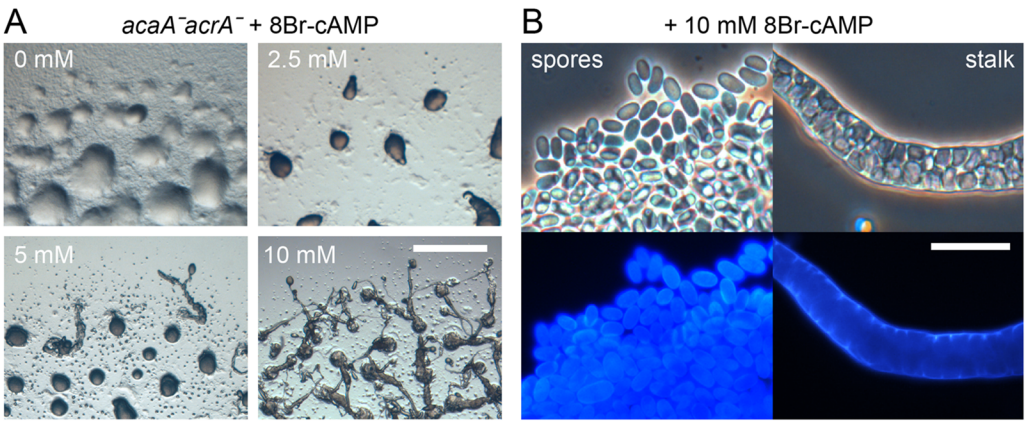

Fig. 5. Induction of fruiting body formation in *acaA⁻acrA⁻* with 8Br-cAMP. (A) Development. *acaA⁻acrA⁻* cells were incubated for 24 h on KK2 agar containing the indicated concentrations of 8Br-cAMP and photographed *in situ*. Scale bar: 1 mm. (B) Spore and stalk cells. The *acaA⁻acrA⁻* structures, developed on agar containing 10 mM 8Br-cAMP, were stained with 0.001% Calcofluor and photographed under phase contrast (top) and epifluorescence (bottom). Scale bar: 20 µm.

AcaA, the pivotal enzyme in the network that generates the secreted cAMP pulses that control *Ddis* aggregation and post-aggregative morphogenesis (Patel et al., 2000; Pitt et al., 1992; Singer et al., 2019).

The loss of AcaA function in *Pvio* did not markedly affect aggregation, fruiting body morphogenesis or spore and stalk cell differentiation. Only stalks appeared somewhat thinner with the individual stalk cells showing a lower width to length ratio (Fig. 1). This suggested that either secreted cAMP played no mayor role in *Pvio* or the role of AcaA was shared with another adenylate cyclase.

P. violaceum AcaA and AcrA are together required for aggregation

Apart from a sporulation defect, *Ddis acrA⁻* mutants also make long thin stalks (Soderbom et al., 1999), while *Pvio acrA* showed similar developmental regulation and cell type specificity as *acaA* (Figs S1 and S3), suggesting that AcrA and AcaA functions may overlap. A *Pvio acrA⁻* mutant showed the same sporulation defect as *Ddis acrA⁻*, but its fruiting bodies were actually more robust than those of wild type (Fig. 2). This was due to the formation of less closely spaced and therefore larger aggregates, suggesting a requirement for AcrA in aggregation centre initiation. A *Pvio acaA⁻acrA⁻* double mutant showed a more severe phenotype. It formed aggregates that never progressed into sorogens but disaggregated instead, only to repeat a cycle of aggregation and disaggregation several times over (Fig. 3). Sorogen and fruiting body formation of *Pvio acaA⁻acrA⁻* was partially restored by developing mutants with the PKA agonist 8Br-cAMP (Fig. 5). While fruiting structures were morphologically aberrant, normal spores and vacuolated stalk cells were formed. *Pvio acaA⁻acrA⁻* fruiting body morphogenesis was also restored by overexpression of either *acaA* or *acrA* (Fig. 4). Since coordination of morphogenetic cell movement is attributed to the propagating cAMP waves that are produced by AcaA, this raised the question, whether cAMP waves are not required for *Pvio* morphogenesis.

P. violaceum development does not require secreted cAMP

To address the question whether cAMP pulses or just secreted cAMP play any role in *Pvio* development, we deleted *pdsA*, which is essential for pulsatile cAMP signalling in *Ddis* (Sucgang et al., 1997) and *carA*, the only cAMP receptor gene that we could detect in the separately assembled genome and transcriptome of *Pvio* (Fig. S7). Both the *carA⁻* and the *pdsA⁻* mutants aggregated normally and formed normal fruiting bodies with mature spore and stalk cells. Unless other unrelated proteins have taken over CarA and PdsA function, this suggests that unlike *Ddis* and *Ppal*, secreted cAMP has no major role in coordination of cell movement or in the induction of prespore differentiation in *Pvio*. This notion was substantiated by *Pvio* aggregation and development proceeding normally in the presence of Sp-cAMPS (Fig. S6). This is a striking result considering that *Pvio* is more closely related to *Ddis*, with its multiple roles for secreted cAMP, than *Ppal*, where deletion of its two *car* genes or *pdsA* gene disrupts fruiting body morphogenesis and of *car* genes also spore differentiation (Kawabe et al., 2002, 2009, 2012). Evidently, not only group 4 but also *Pvio* underwent significant innovations in developmental control.

Which secreted signal(s) might have taken over the roles of secreted cAMP in *Pvio*? Here the *Pvio* chemoattractant glorin comes first to mind, since it was also reported to trigger gene expression in early *Ppal* development (Asghar et al., 2011). However, Dictyostelids are known to synthesize a range of other secondary metabolites that affect cell differentiation (Araki and Saito, 2019; Kikuchi et al., 2013; Kondo et al., 2019; Saito et al., 2022; Sasaki et al., 2020; Tsujioka et al., 2004) and focusing on one or a few compounds is therefore premature.

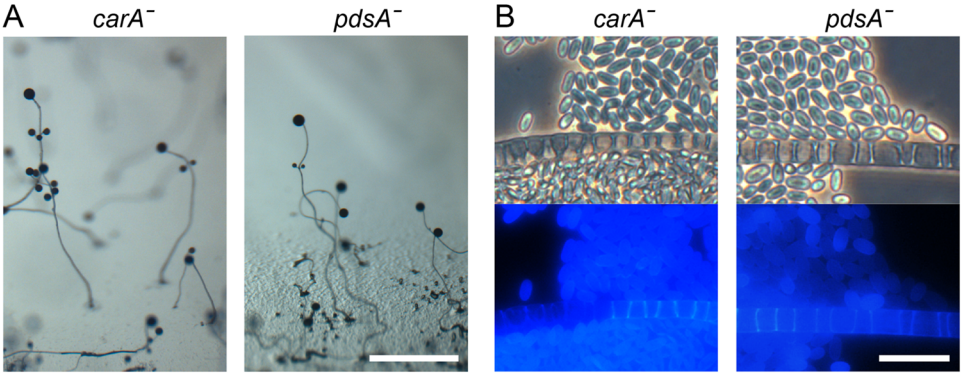

Fig. 6. Development and differentiation of *carA⁻* and *pdsA⁻ Pvio* cells. (A) Fruiting body morphology. Scale bar: 1 mm. (B) Spore and stalk cells. Mature fruiting structures were stained with 0.001% Calcofluor *in situ* and photographed under phase contrast (top) and epifluorescence (bottom). Scale bar: 20 µm.

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Taxon group-specific functionalization of the Dictyostelid adenylate cyclases

Comparative analysis of adenylate cyclase function across Dictyostelia highlight considerable refunctionalisation of AcaA, AcrA and AcgA in the course of dictyostelid evolution. AcaA is essential in *Ddis* for producing the secreted cAMP pulses that organize aggregation and post-aggregative morphogenesis (Patel et al., 2000; Pitt et al., 1992), although a *Ddis acrA*$^-$*acgA*$^-$*acaA*$^-$ mutant that overexpresses PKA can still form mounds at high cell density (Hirose et al., 2021). *Ddis* AcaA also acts intracellularly in response to the stalk-inducer c-di-GMP to activate PKA and thereby stalk maturation (Chen and Schaap, 2012; Chen et al., 2017). However, apart from a reduction in stalk thickness, *Pvio acaA*$^-$ mutants showed no developmental abnormalities. *Ppal aca1*$^-$ cells also form fruiting bodies with thinner stalks. However, even knock-outs in all three *Ppal aca* genes still form some fruiting bodies after a long delay (Kawabe and Schaap, 2022).

AcrA is required for robust stalk formation in *Ddis* and has an overlapping role with AcgA in induction of prespore differentiation and spore maturation (Alvarez-Curto et al., 2007; Soderbom et al., 1999). AcgA on its own mediates inhibition of spore germination by high osmolarity in the *Ddis* spore head (Van Es et al., 1996). However, neither AcrA nor AcgA or both together are essential for *Ppal* prespore and spore differentiation. They do have overlapping roles in mediating stress-induced encystation in *Ppal* and inhibition of spore and cyst germination by high osmolarity (Kawabe et al., 2015). Induction of prespore differentiation and spore maturation is dependent on both Cars and PKA in *Ppal* (Funamoto et al., 2003; Kawabe et al., 2009, 2015) as it is in *Ddis* (Hopper et al., 1993; Schaap and Van Driel, 1985). We surmised that in the *Ppal acrA*$^-$*acgA*$^-$ cells, any or all of the three *Ppal Aca* enzymes may provide cAMP for spore differentiation.

Similar to *Ddis* AcrA (Soderbom et al., 1999), *Pvio* AcrA is required for the differentiation of stable spores (Soderbom et al., 1999) (Fig. 2). *Pvio AcrA* is additionally required for efficient aggregation centre initiation and has an overlapping role with *AcaA* in the formation of stable aggregates. For these roles both AcaA and AcrA act upstream of PKA, since stable aggregation and fruiting body formation are restored in *acaA*$^-$*acrA*$^-$ mutants by 8Br-cAMP (Fig. 5). The distinction in *Ddis* between roles of AcaA in mostly extracellular Car activation, and roles of AcrA and AcgA in mostly PKA activation is therefore blurred in *Ppal* and *Pvio*.

Compared to other Amoebozoa, such as *Physarum polycephalum*, *Protostelium fungivorum* and *Acanthamoeba castellani* with, respectively, 64, 52 and 67 adenylate cyclases each (Clarke et al., 2013; Hillmann et al., 2018; Schaap et al., 2015), the number of adenylate cyclases in Dictyostelia is low. It is possible that in the unicellular ancestor of Dictyostelia the roles of their three adenylate cyclases were not highly specialized, i.e. they all responded to environmental stressors to induce the transition of amoebas into dormant cysts. In the newly emerging Dictyostelid taxon groups, the enzymes and their regulation may then have evolved independently to take on group-specific developmental roles.

MATERIALS AND METHODS

Growth and development

P. violaceum QSvi11, (Pvio) (Kalla et al., 2011), gift from J. E. Strassmann (Washington University in St. Louis, USA) was routinely grown in KK2 (16 mM KH_2PO_4 and 4 mM K_2HPO_4), containing autoclaved *Klebsiella aerogenes* (*K.aer*) (final OD_{600}=8.5) and 10% HL5 shaken at 150 rpm. For some experiments cells were grown in association with *Escherichia coli* on 1/5th SM agar (Formedium, UK). For multicellular development, cells were harvested from growth media and spread at 10^6 cells/cm^2 on KK2 agar (1.5% agar in KK2), incubated at 4°C overnight and then at 22°C until the desired developmental stage had been reached.

DNA constructs and transformation

AcaA and *acrA* promoter-*lacZ* constructs and analysis

To construct a gene fusion of the promoter of *Pvio acaA* (Pvio_g2213, NCBI id.: KAF2076473) and *lacZ*, a fragment comprising the full 1.8 kb *acaA* 5′intergenic region and 0.1 kb 5′ coding sequence was amplified from *Pvio* gDNA, using primer pair Pv-ACA-P51 K and Pv-ACA-P31B (Table S1) that harbour *Kpn*I and *Bam*HI sites, respectively. After *Kpn*I/*Bam*HI digestion, the fragment was ligated into *Kpn*I/*Bam*HI digested pDdGal16 (Harwood and Drury, 1990), yielding vector pPv-acaA-LacZ.

To construct an *acrA_LacZ* fusion, a fragment from −822 to +125 nt relative to the start ATG of *acrA* (Pvio_g1249, KAF2077476) and containing the full 5′ intergenic region was amplified from *Pvio* gDNA, using primers Pv-ACB-P51X and Pv-ACB-P31B (Table S1) that harbour *Xba*I and *Bam*HI sites, respectively. After digestion the fragment was ligated into *Xba*I/*Bam*HI digested pDdGal16, yielding vector pPv-acrA-LacZ.

Both plasmids were validated by DNA sequencing and transformed into *Pvio* cells by electroporation. Transformants were selected at 50 µg/ml G418 on growth plates with G418 resistant *E. coli* (Narita et al., 2020). Transformed cells were developed on dialysis membrane supported by KK2 agar and β-galactosidase activity was visualised with X-gal in developing structures as described previously (Dingermann et al., 1989).

Knockout constructs for *Pvio acaA*, *acrA*, *carA* and *pdsA*

To disrupt *Pvio acaA*, an *acaA* fragment was amplified from *Pvio* gDNA using primers Pv-aca-51S2 and Pv-aca-31K that harbour *Sac*II and *Kpn*I sites (Table S1), respectively. After digestion, the fragment was cloned into *Sac*II/*Kpn*I digested pBluescript SK+, which was next digested with *Bam*HI and *Sal*I. The LoxP-NeoR cassette was excised from pLoxNeoIII (Kawabe et al., 2012) with *Bam*HI/*Sal*I and ligated into the digested acaA-pBluescript vector. This yielded pPv-acaA-KO in which LoxP-NeoR was flanked by 1870 bp of 5′UTR and 5′ *acaA* sequence and 1825 bp of 3′ *acaA* sequence (Fig. S2A).

To disrupt *Pvio acrA*, two *acrA* fragments, KO-A and KO-B, were amplified from *Pvio* genomic DNA using primer pair Pv-ACB-51K/Pv-ACB-31S with *Kpn*I and *Sac*II sites for KO-A and Pv-ACB-52 K/Pv-ACB-32 with a *Kpn*I site for KO-B (Table S1). After *Kpn*I/*Sac*II digestion, KO-A was ligated into *Kpn*I/*Sac*II digested pBluescript SK+, which was next digested with *Kpn*I/*Xba*I and ligated to LoxP-NeoR, which was excised from pLoxNeoIII with *Kpn*I/*Xba*I. Fragment KO-B was digested with *Kpn*I/*Xho*I and cloned into this vector, yielding vector pPv-acrA-KO with LoxP-NeoR flanked by 1483 bp 5′*acrA* sequence and 1373 bp 3′*acrA* and 3′UTR sequence (Fig. S2B).

The linearized KO vectors were transformed in *Pvio* (Narita et al., 2020). Genomic DNA was isolated from G418 resistant clones and screened by PCR to diagnose gene disruption by homologous recombination (Fig. S2). To generate a double *acaA*$^-$*acrA*$^-$ knock-out, *acaA*$^-$ cells were transformed with pA15NLS.Cre for transient expression of Cre-recombinase (Faix et al., 2004). G418 sensitive clones were selected and transformed with pPv-acrA-KO and diagnosed for *acrA* gene disruption by PCR (Fig. S2B).

To disrupt *Pvio carA* (Pvio_g6080, KAF2072602), two *carA* fragments, A and B, were amplified from *Pvio* gDNA using primer pair Pv-cAR-51K/Pv-cAR-31C with *Kpn*I and *Cla*I sites for A and Pv-cAR-52B/Pv-cAR-32X with *Bam*HI and *Xba*I sites for B (Table S1). After digestion with *Kpn*I and *Cla*I, fragment A was ligated into *Kpn*I/*Cla*I digested pLoxNeoIII. Fragment B was digested with *Bam*HI/*Xba*I and cloned into this vector, yielding vector pPv-carA-KO in which LoxP-NeoR is flanked by 1183 bp 5′UTR and 5′*carA* sequence and 1068 bp 3′ *carA* and 3′UTR sequence (Fig. S5A).

To disrupt *Pvio pdsA* (Pvio_g5708, KAF2072968), two *pdsA* fragments, A and B with internal *Hind*III and *Sac*I sites, respectively, were amplified from *Pvio* gDNA using primer pair Pv-pdsA-51K with *Kpn*I site and Pv-pdsA-31 for A and Pv-pdsA-52B with *Bam*HI site and Pv-pdsA-32 for B (Table S1). Fragment B was digested with *Bam*HI/*Sac*I and ligated into *Bam*HI/*Sac*I digested pLoxNeoIII. Fragment A was digested with *Kpn*I/

*Hind*III and cloned into this vector, yielding vector pPv-pdsA-KO that contained 1391 bp 5′UTR and 5′*pdsA* sequence and 1205 bp 3′*pdsA* and 3′UTR sequence (Fig. S5B).

Complementation of adenylate cyclase knock-outs with *acaA* or *acrA*

To express *Pvio acaA* from its own promoter, a 5833 bp segment containing the *Pv-acaA* promoter, coding and terminator regions was amplified from gDNA in two fragments, A and B, using primer set Pv-aca-P51K (with *Kpn*I site) and Pv-aca-C31 for A and Pv-aca-C51 and Pv-aca-31C (with *Cla*I site) for B. The fragments were cloned into pCR-BluntII-TOPO for sequence validation. Using an *acaA* internal *Bam*HI site, fragment A was isolated from its TOPO plasmid using *Kpn*I and *Bam*HI and ligated into the *Kpn*I/*Bam*HI digested plasmid that contained fragment B, thus reconstructing the entire 5.8 kb *acaA* genomic segment. This segment was excised with *Kpn*I/*Cla*I and ligated into *Kpn*I/*Cla*I digested vector pHygTm(plus)/pG7 (http://dictybase.org/db/cgi-bin/dictyBase/SC/plasmid_details.pl?id=453), which contains a hygromycin resistance cassette, yielding vector pPv-acaA-Exp.

To express *Pvio acrA* from its own promoter, a segment containing the *acrA* coding region, part of its promoter and the terminator were amplified from gDNA using primers Pv-ACB-P51X/Pv-ACB-31S. The fragment was digested with *Spe*I/*Sac*II and cloned into pBluescript SK+ for sequence validation, yielding plasmid pBs-PvAcrA. The remaining part of the promoter region was excised with *Xba*I/*Sma*I from pPv-acrA-LacZ (see above) and ligated into the *Xba*I/*Sma*I digested vector pHygTm(plus)/pG7, which was subsequently digested with *Sma*I and *Spe*I. The PvAcrA fragment from pBs-PvAcrA was excised with *Spe*I/*Hpa*I and ligated into the *Spe*I/*Sma*I digested vector, yielding pPv-acrA-Exp, which now harboured a 6.4 kb region encompassing the *acrA* promoter, coding region and terminator. pPv-acrA-Exp was introduced into both *acrA*$^-$ and *acaA*$^-$/*acrA*$^-$ cells by electroporation and pPv-acaA-Exp into *acaA*$^-$/*acrA*$^-$ only. Transformants were incubated with autoclaved *Klebsiella aerogenes* in 10% HL5 with 30 µg/ml of hygromycin in petri dishes for 48 h, and next distributed with *E. coli* on 1/5th SM plates supplemented with 30 µg/ml of hygromycin. The plasmids and knock-out cell lines prepared for the study are in the *Dictyostelium* Stock Center http://dictybase.org/StockCenter/StockCenter.html.

Data analysis

Quantitative data were collected in Excel (Microsoft). Statistical analysis and graph preparation was performed in Sigmaplot v14.5 (Systat Software, Inc.).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.K., P.S.; Methodology: Y.K.; Validation: Y.K., P.S.; Investigation: Y.K.; Data curation: P.S.; Writing - original draft: Y.K.; Writing - review & editing: P.S.; Project administration: P.S.; Funding acquisition: P.S.

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Data availability

All data generated in this work are presented in the main and supplemental figures. The plasmids and knock-out cell lines prepared for the study are deposited in the *Dictyostelium* Stock Center http://dictybase.org/StockCenter/StockCenter.html.

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