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Effects of deletion of the receptor CrlA on *Dictyostelium* aggregation and MPBD mediated responses are strain-dependent and not evident in strain Ax2

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ABSTRACT

The polyketide MPBD (4-methyl-5-pentylbenzene-1, 3-diol) is produced by the polyketide synthase SteelyA (StlA) in *Dictyostelium discoideum*. MPBD is required for appropriate expression of cAMP signalling genes involved in cell aggregation and additionally induces the spore maturation at the fruiting body stage. The MPBD signalling pathway for regulation of cell aggregation is unknown, but MPBD effects on sporulation were reported to be mediated by the G-protein coupled receptor CrlA in *D. discoideum* KAx3. In this study, we deleted the *crlA* gene from the same parental strain (Ax2) that was used to generate the MPBD-less mutant. We found that unlike the MPBD-less mutant, Ax2-derived *crlA*$^-$ mutants exhibited normal cell aggregation, indicating that in Ax2 MPBD effects on early development do not require CrlA. We also found that the Ax2/*crlA*$^-$ mutant formed normal spores in fruiting bodies. When transformed with PkaC, both Ax2 and Ax2/*crlA*$^-$ similarly responded to MPBD *in vitro* with spore encapsulation. Our data make it doubtful that CrlA acts as the receptor for MPBD signalling during the development of *D. discoideum* Ax2.

Keywords: *Dictyostelium*, polyketide, MPBD, sporulation, aggregation

INTRODUCTION

Dictyostelium discoideum is a eukaryotic microorganism that inhabits the soil as single cell amoebae. When their bacterial food is exhausted, starving cells aggregate by means of chemotaxis to secreted cAMP and form multicellular mounds, which after a migratory slug stage, transform into fruiting bodies, consisting of a stalk, spore mass and basal disc. The spore cells become encapsulated and enter a dormant state (Konijn *et al.*, 1967, Loomis, 1975, Kessin, 2001, Kay & Thompson, 2009).

Polyketides, synthesised by polyketide synthases (PKSs), are secondary metabolites of various organism, which display a range of biological activities (Rideout *et al.*, 1979, Funa *et al.*, 2006, Schindler & Nowrousian, 2014). The *D. discoideum* genome has more than 40 genes encoding PKSs (Eichinger *et al.*, 2005, Zucko *et al.*, 2007) and some polyketide compounds have been identified. DIF-1 (Differentiation Inducing Factor-1) is the best studied polyketide in *D. discoideum*; its backbone is synthesised by the polyketide synthase SteelyB and then modified by the halogenase ChlA and the methyltransferase DmtA to produce DIF-1 (Thompson & Kay, 2000, Austin *et al.*, 2006, Neumann *et al.*, 2010). DIF-1 induces the basal disc and lower cup of the fruiting body, but also modulates chemotaxis to cAMP (Saito *et al.*, 2008, Kuwayama & Kubohara, 2009). Dictyoquinone is a polyketide, which has prespore cell inducing activity in *D. discoideum* and triggers the aggregation process in another Dictyostelid

Polysphondylium violaceum (Oohata *et al.*, 2009, Takaya *et al.*, 2014).

MPBD (4-methyl-5-pentylbenzene-1, 3-diol) is produced by the PKS SteelyA. MPBD was originally identified as a stalk cell inducing factor and was also shown to have spore inducing activity *in vitro* (Saito *et al.*, 2006, Narita *et al.*, 2011). In addition, MPBD induces spore maturation in fruiting bodies (Sato *et al.*, 2013, Narita *et al.*, 2014). Previous studies suggest that MPBD is a component of the SDF-1 (Spore Differentiation Factor-1) signalling cascade (Anjard *et al.*, 2011). SDF-1 is a phospho-peptide, which induces spore encapsulation in sporogenous cells that overexpress PkaC. Furthermore, SDF-1 can induce stalk cell differentiation in *D. discoideum* strain V12M2 cells *in vitro* (Anjard *et al.*, 1998). In the SDF-1 pathway, MPBD is proposed to bind to the G-protein coupled receptor CrlA (Raisley *et al.*, 2004), coupled to Gα1 (Pupillo *et al.*, 1989), which then blocks the inhibitory effect of glycogen synthase kinase GskA (Harwood *et al.*, 1995) on release of the SDF-1 precursor.

We recently found that in addition to its role in sporulation, MPBD is also essential for early development, where it induces competence for aggregation of starved cells by upregulating the expression of key genes involved in chemotactic cAMP signalling (Narita *et al.*, 2014). The mode of action of MPBD on induction of aggregation competence in early development has not been investigated. However, the genes required for SDF-1 signalling (*stlA*, *crlA*, *gpaA* and *gskA*) are present in early development (Rot *et al.*, 2009) and CrlA may therefore also mediate the effects of MPBD at this stage. To validate this

supposition, we deleted the *crlA* gene in *D. discoideum* Ax2, the parent of the MPBD-less *stlA*$^-$ mutant, because the earlier *crlA* knock-out was generated in a different genetic background (KAx3) (Raisley *et al.*, 2004).

However, we found no effect of CrlA deletion on MPBD effects on early development and upon further study also no effects on MPBD induction of sporulation. It therefore appears that CrlA does not mediate the effect of MPBD in strain Ax2.

MATERIALS AND METHODS

Strains, cell culture and development

Dictyostelium discoideum Ax2 and KAx3 strain were grown in an axenic medium (HL-5) at 22^{o}C (Watts & Ashworth, 1970). The *stlA*$^-$ strain was described previously (Austin *et al.*, 2006) and the *crlA*$^-$ strain (Strain ID: DBS0235627, KAx3-derivative) was obtained from Dicty Stock Center (Raisley *et al.*, 2004). Both strains were cultured in HL-5, supplemented with 10 µg/ml blasticidin S. To create *pkaC*-overexpressing strains, cells were transformed with the pK-Neo vector (Anjard *et al.*, 1992) by electroporation and the transformants were selected at 20 µg/ml G418. For multicellular development, cells cultured shaking in HL-5 were harvested in log phase, washed twice with phosphate buffer (PB) (2.7 mM Na_2HPO_4/10.7 mM K_2HPO_4, pH 6.2), plated at 10^6 cells/cm^2 on PB agar plates (1.5^o/$_o$ agar in PB) or on nitrocellulose filters, supported by agar, and

incubated at 22^{o}C. For submerged development, axenically grown cells were washed twice with PB, and then incubated in 1 ml PB at 2 × 10^5 cells/cm^2 in 6-well tissue culture plates.

Gene disruption of CrlA in Ax2 strain

For *crlA* gene disruption in *D. discoideum* Ax2, two DNA fragments of 688 bp and 972 bp were amplified from Ax2 genomic DNA by PCR using primer pairs CrlA Frag1 F/CrlA Frag1 R and CrlA Frag2 F/CrlA Frag2 R (Table. S1) and LA Taq polymerase (Takara) or Q5 Hot Start High-Fidelity DNA Polymerase (New England BioLabs), and then subcloned into vector pCR4-TOPO (Invitrogen). The fragments were excised from the vectors using KpnI/HindIII for the 688 bp fragment 1 and PstI/BamHI for the 972 bp fragment 2 (Fig. S1) and both fragments were inserted into vector pLPBLP to generate the knockout vector pCrlA-KO. Ax2 cells were transformed with the KpnI/BamHI-digested pCrlA-KO vector by electroporation. After selection at 10 μg/ml blasticidin S, the knockout of *crlA* gene by homologous recombination was diagnosed by PCR of genomic DNA of transformed clones and by reverse transcription PCR of isolated RNA (Fig. S1).

Sporulation and spore viability assays

Cells were developed at 10^6 cells/cm^2 on a qualitative filter paper (ADVANTEC) cut into 1.5 cm square or a quarter of nitrocellulose filter (0.45 μm

pore size, Black gridded, 47 mm diameter, Millipore) placed on PB agar plates. When using KAx3 strains, agar plates with 4-fold concentrated PB were used, because KAx3 slugs migrated away from the filters on normal PB agar. After 2 days, when fruiting bodies had formed, the filters were transferred to 1 ml PB and shaken vigorously. Spores were both directly stained with $0.001^{o}/_{o}$ calcofluor and counted using a fluorescence microscope, and treated with $0.5^{o}/_{o}$ Triton X-100 for 10 min and then stained and counted. Detergent-resistant spores were calculated as the ratio of the number of post-detergent treatment spores to pre-detergent treatment spores.

For examining spore viability, 50 detergent treated spores were plated on a 1/5th SM agar plate with *Klebsiella aerogenes,* and the number of plaques appearing after 3 to 4 days was scored. Spore viability was calculated as the ratio of the number of plaques to the initial number of spores plated on plates.

In vitro sporulation assay

The *in vitro* sporulation assay using sporogenous *pkaC*-overexpressing strains was performed as described previously (Kay, 1987, Anjard *et al.*, 1998) with a few modifications. Axenically grown cells in shaking culture were harvested in the log phase, washed twice in KK_2 buffer (16.5 mM KH_2PO_4 and 3.9 mM K_2HPO_4, pH 6.1), and then resuspended at 10^5 cells/ml in spore salts (10 mM MES; pH 6.2, 20 mM NaCl, 20 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 100 units penicillin and 100 µg/ml streptomycin) with 5 mM cAMP. Two ml aliquots of

cell suspension were dispensed into 35 mm diameter dishes, yielding 2×10^3 cells/cm^2. After incubation at 22^{o}C for 24 hours, MPBD was added and spores and amoeboid cells were scored 2 hours later by microscopy. At least 200 cells were counted and each assay was performed in duplicate.

RESULTS

Creation of Ax2-derived crlA mutant

To examine the relationship between MPBD and CrlA in the same genetic background, we disrupted the *crlA* gene to delete transmembrane domains four to six of CrlA in *D. discoideum* strain Ax2, which is also the parental strain of the MPBD-less *stlA*$^-$ mutant. Three independent knockout clones were used in this study (Fig. S1).

Firstly, we checked cell growth of Ax2/*crlA*$^-$ mutants, because in KAx3, CrlA acts as a negative regulator of cell growth (Raisley *et al.*, 2004). In KAx3/*crlA*$^-$ mutants have a shorter doubling time and achieve a two times higher cell density in stationary phase than wild-type KAx3. Figure 1 shows that the Ax2/*crlA*$^-$ mutants showed similar cell growth as Ax2. The doubling time of Ax2 cells was 9.3 ± 0.3 hours during the exponential phase, while that of three Ax2/*crlA*$^-$ mutants were 9.3 ± 0.7, 9.1 ± 0.1, and 9.6 ± 0.3 hours, respectively. The three clones also reached the same stationary phase cell densities as Ax2, indicating that CrlA is not a negative regulator of cell growth in Ax2.

Ax2-derived crlA⁻ cells exhibit normal cell aggregation

To assess whether CrlA mediates MPBD signalling during early development, Ax2, Ax2/*crlA*⁻ and *stlA*⁻ cells were developed under submerged conditions and on nitrocellulose filters, and aggregation timing and morphology were observed. When submerged, Ax2/*crlA*⁻ cells aggregated as rapidly as Ax2 cells and formed similar large streaming aggregates, while aggregation of *stlA*⁻ cells was about 3 hours delayed, with much reduced streaming and smaller aggregation territories (Fig. 2A). When developed on nitrocellulose filters, Ax2/*crlA*⁻ and Ax2 aggregates were again of similar size, while *stlA*⁻ aggregates were much smaller (Fig. 2B). These observations were confirmed when cells were developed directly on PB agar plates (data not shown). Thus, unlike the MPBD-less *stlA*⁻ mutant, the Ax2/*crlA*⁻ mutant shows no obvious defects during early development. These results make it unlikely that CrlA acts as a receptor for MPBD during early development.

CrlA is not required for spore formation in Ax2 strain

Spore formation was previously reported to be impaired in the KAx3/*crlA*⁻ mutant as well as the *stlA*⁻ mutant, with both the percentage of detergent-resistant spores and spore viability being reduced in both mutants, suggesting that CrlA is the receptor of MPBD (Anjard *et al.*, 2011). To validate these findings for Ax2, we examined the spore formation in our Ax2/*crlA*⁻ mutants.

While the *stlA*$^-$ mutant makes fruiting bodies with transparent (glassy) spore heads, the Ax2/*crlA*$^-$ mutants form fruiting bodies which have normal “milky” spore heads, similar to Ax2 (Fig. 3A). Both Ax2 and Ax2/*crlA*$^-$ spore heads almost completely consist of calcofluor-positive elliptical spores, while the *stlA*$^-$ spore heads contain many calcofluor-negative spores and amoebas (Fig. 3B). The percentage of detergent-resistant spores and the percentage of spores that produced viable offspring were reduced to 59 ± 2.0$^o/_o$ and 49 ± 11$^o/_o$, respectively, in the *stlA*$^-$ mutant. However, there were no significant differences in detergent resistance and spore viability between Ax2 and Ax2/*crlA*$^-$ mutants (Fig. 3C, 3D). These results indicate that unlike *stlA*$^-$ mutant, the Ax2/*crlA*$^-$ mutants formed normal encapsulated and viable spores. This implies that also MPBD induction of sporulation is not mediated by CrlA.

Although the Ax2/*crlA*$^-$ mutants were able to form normal spores, the KAx3/*crlA*$^-$ mutant was reported to show deficient spore formation and to be insensitive to MPBD (Anjard *et al.*, 2011). To evaluate whether the different phenotypes of the *crlA* knock-outs in the Ax2 and KAx3 backgrounds are due to variations in laboratory procedures, we compared formation of encapsulated and detergent resistant spores in fruiting bodies of Ax2, *stlA*$^-$, Ax2/*crlA*$^-$, KAx3, and KAx3/*crlA*$^-$ strains. Figure 4A shows that unlike the Ax2/*crlA*$^-$ mutant, the sporulation efficiency was significantly reduced in the KAx3/*crlA*$^-$ mutant as well as the *stlA*$^-$ mutant, which meant both strains made less spores in the fruiting bodies. However, there was a difference between *stlA*$^-$ and KAx3/*crlA*$^-$ spores;

the *stlA*$^{-}$ spores were also less detergent resistant, while the KAx3/*crlA*$^{-}$ spores showed almost normal detergent resistance (Fig. 4B). These results indicate that effects of CrlA inactivation on sporulation is strain-dependent.

Cells lacking CrlA in Ax2 strain can respond to MPBD

Unlike MPBD-less *stlA*$^{-}$ mutants, Ax2/*crlA*$^{-}$ mutants show normal sporulation and spore viability, suggesting that CrlA is not required for MPBD induction of sporulation. To validate this further we tested whether cells lacking CrlA respond to MPBD in a sporulation bioassay. According to the standard procedure to generate sporogenous strains (Anjard *et al.*, 1992), we transformed the pK-Neo vector into Ax2 or Ax2/*crlA*$^{-}$ cells to create Ax2/K and Ax2-*crlA*$^{-}$/K, respectively. We next used these PkaC overexpressing strains to test whether MPBD induced sporulation *in vitro*. Without MPBD, approximately $10^{o}/_{o}$ of Ax2/K cells differentiated into spore cells. MPBD was ineffective at a 1 nM, but increased spore differentiation of Ax2/K cells to 20-$25^{o}/_{o}$ at 10-200 nM MPBD (Fig. 5A). Ax2-*crlA*$^{-}$/K cells showed similar induction of sporulation as Ax2/K cells by 10 nM or 100 nM MPBD. This indicates that Ax2 cells that lack CrlA still respond to MPBD for spore differentiation (Fig. 5B). CrlA therefore either does not mediate MPBD induction of sporulation, or acts redundantly with another receptor in Ax2.

DISCUSSION

Studies of a *crlA*$^-$ mutant in *D. discoideum* strain KAx3 indicated that CrlA acts as receptor for MPBD and component of the SDF-1 signalling cascade that triggers terminal differentiation (Anjard *et al.*, 2011). We initially aimed to investigate involvement of CrlA in effects of MPBD on early development and generated a second *crlA*$^-$ mutant in strain Ax2, which was also the parent of the MPBD-less mutant *stlA*$^-$. The Ax2/*crlA*$^-$ mutant did not show the defects in early development that we found earlier in *stlA*$^-$. Unlike *stlA*$^-$ mutants, the Ax2/*crlA*$^-$ mutants sporulated normally in fruiting bodies and also responded normally to MPBD in an *in vitro* sporulation assay. It is therefore doubtful that CrlA acts as the receptor for MPBD signalling during early or late development of *D. discoideum* Ax2.

We propose that the discrepancy with the earlier results using a KAx3/*crlA*$^-$ strain are caused by the genetic difference between Ax2 and KAx3 strains. Both strains are derived from the *D. discoideum* NC4 wild-type, but were chemically mutagenized to allow growth in axenic media. The KAx3 genome contains a large duplication in chromosome 2 that both NC4 and Ax2 lack. In addition, small duplications or deletions on some chromosomes have appeared over time in different laboratory stocks of the same strain (Bloomfield *et al.*, 2008). Because the Ax2 genome could also have accumulated mutations, it could contain mutations that act epistatically with *crlA* or genes in the CrlA pathway. Thus, these subtle different genotypes might lead to different phenotypes of knockout mutants in Ax2 and KAx3. In fact, *gskA* knockout mutants in Ax2 or

Ax3 parents also show major differences in phenotype (Harwood *et al.*, 1995, Schilde *et al.*, 2004). GskA is described as the downstream component of CrlA in SDF-1 signalling cascade (Anjard *et al.*, 2011) and its involvement therefore also requires further study.

Our results also indicate differences in the regulation of *Dictyostelium* cell growth and/or development between Ax2 and KAx3. Earlier data show increased doubling time and stationary phase cell densities in the KAx3/*crlA*$^-$ mutant, indicating that CrlA is a negative regulator of cell growth in axenic medium. The growth "defect" is cell-autonomous, suggesting that the KAx3/*crlA*$^-$ mutant cannot sense a secreted signal that regulates cell proliferation (Raisley *et al.*, 2004). Ax2/*crlA*$^-$ mutants, however, exhibit similar cell growth as wild type Ax2 (Fig. 1), indicating that such a signal is unnecessary for growth regulation in Ax2.

CrlA has an additional cell-autonomous role in prestalk differentiation in KAx3 because when developed in chimeras with KAx3 cells, KAx3/*crlA*$^-$ cells will sort out to the prespore region of slugs (Raisley *et al.*, 2004). We performed a similar experiment with GFP-labelled Ax2/*crlA*$^-$ and Ax2 cells, but found that Ax2/*crlA*$^-$ cells were evenly distributed in the chimeric slug (data not shown). Furthermore, the KAx3/*crlA*$^-$ strain was reported to form larger aggregates with delayed tip formation compared to the wild type KAx3 (Raisley *et al.*, 2004). These phenomena, however, were not observed when comparing Ax2/*crlA*$^-$ to the Ax2 parent. In short, all reported phenotypic consequences of *crlA* knock-out in KAx3 are not found in Ax2. One explanation, other than the difference of

genotypes, could be redundancy of CrlA function with another receptor in Ax2 that is missing in KAx3. Although we did not detect a *crlA*-like mRNA in the Ax2/*crlA*$^-$ mutant (Fig. S1), it is possible that there is redundancy between CrlA and more distantly related receptor. Also, different constructs used for creating the mutants might lead to phenotypic differences in Ax2 and KAx3 backgrounds. The construct used in KAx3 was designed to disrupt the *crlA* by insertion of the blasticidin resistance (Bsr) cassette at a unique site (Raisley *et al.*, 2004), while our construct replaces part of *crlA* with the Bsr cassette (Fig. S1). This difference in gene targeting could also affect phenotypes in each mutant.

Although our results make it doubtful that CrlA acts as the main receptor for MPBD signalling in Ax2, we cannot exclude that CrlA is the MPBD receptor in KAx3. In our experiments the sporulation defective phenotype of KAx3/*crlA*$^-$ is different from the MPBD-less phenotype (Fig. 4), suggesting that the sporulation defect of the KAx3/*crlA*$^-$ acts in parallel to the MPBD pathway.

In conclusion, we show that unlike in KAx3, CrlA does not act as the sole receptor for MPBD in Ax2. In future research, we aim to identify the missing MPBD receptor in Ax2.

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CONFLICT OF INTEREST

None declared.

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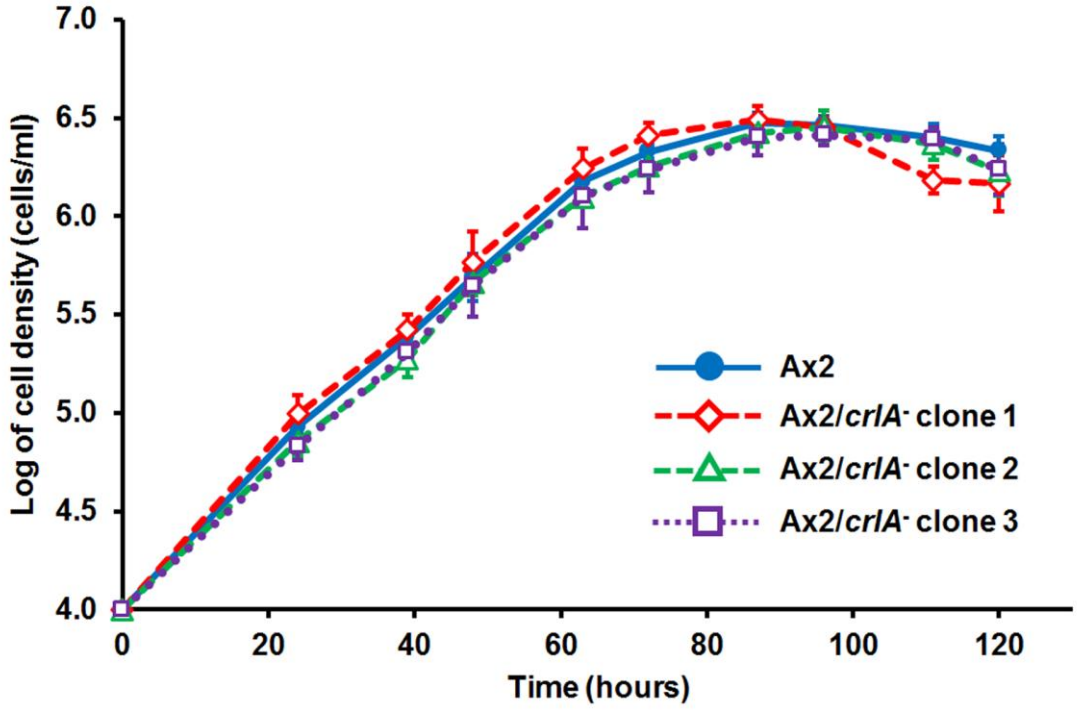

Figure 1. Axenic growth of Ax2/*crlA*$^-$ and Ax2 cells.

Cells were shaken at 200 rpm at 22^{o}C at an initial starting density of 10^5 cells/ml in HL-5 and counted using a haemocytometer at the indicated times. Means and SD of three independent experiments are presented.

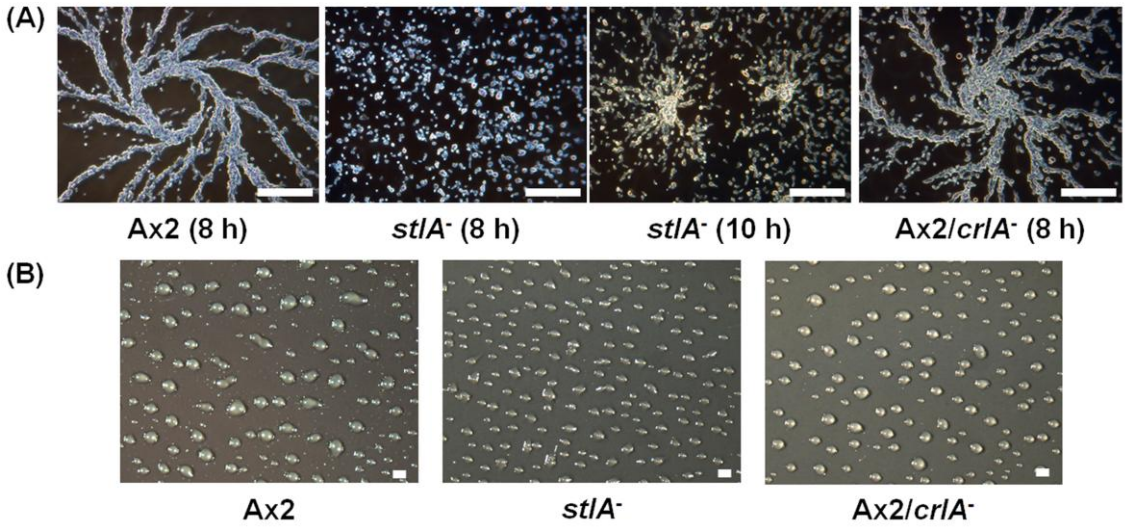

Figure 2. Early development of Ax2/*crlA*$^-$ and MPBD-less mutants.

(A) Cells were plated at 2 × 10^5 cells/cm^2, submerged in PB, and photographed at the indicated time points. Bars: 500 μm. (B) Cells were developed on nitrocellulose filters, supported by PB agar at 1 × 10^6 cells/cm^2. Aggregates were photographed at the moment that each strain had completed aggregation. Three clones of the Ax2/*crlA*$^-$ mutant showed the same aggregate size. Bars: 500 μm.

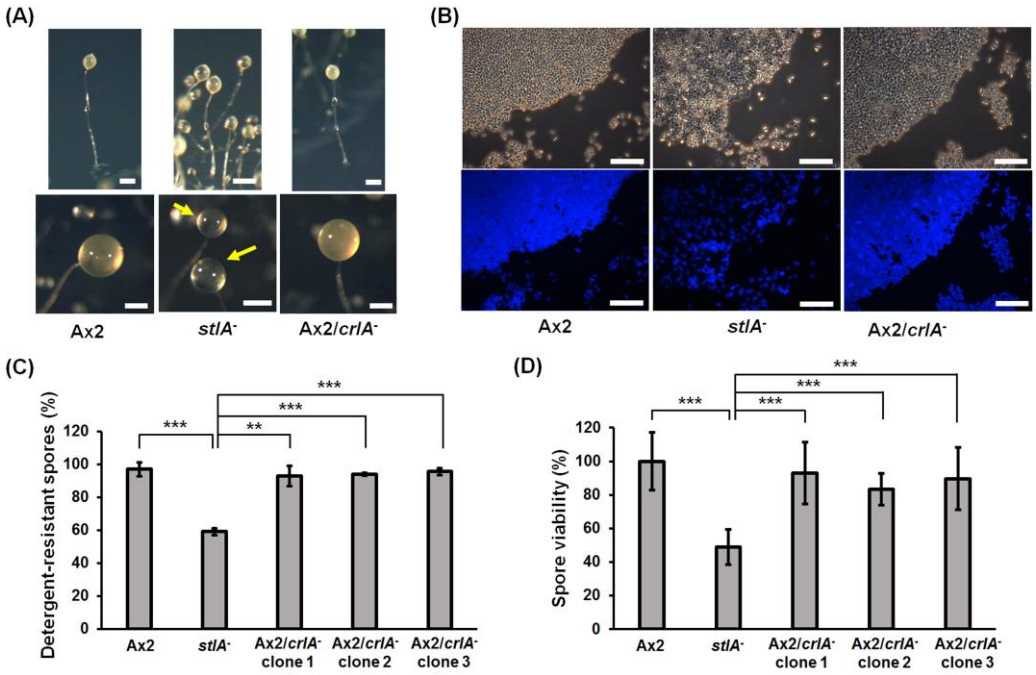

Figure 3. Fruiting body and spore formation in Ax2/*crlA*$^-$ mutants.

(A) Cells were developed on PB agar plates at 10^6 cells/cm^2. After completing fruiting body formation, fruiting bodies (top panels) and spore heads (bottom panels) of each strain were photographed. Bars: 500 μm (top panels) and 200 μm (bottom panels). (B) Spores were collected from mature fruiting bodies and stained by 0.001$^o/_o$ calcofluor. The spores were observed under phase contrast (top panels) and UV illumination (bottom panels). The same phenomena were observed for each clone of Ax2/*crlA*$^-$ mutants. Bars: 50 μm. (C) The percentage of detergent-resistant spores. Spores were collected from sori after 2 days from starvation and counted, excluding unencapsulated amoeba-like cells. Spores were next treated with 0.5$^o/_o$ Triton X-100 and counted once more. Detergent-resistant spores were calculated as the percentage of post-treatment

spores to pre-treatment spores. Bars represent means and SD of three experiments, performed in duplicate. (D) Spore viability. After detergent treatment, 50 spores were plated on 1/5th SM agar plates with *K. aerogenes* and emerging plaques were counted. Spore viability was calculated as the ratio between emerging plaques and plated spores and this value was expressed as percentage of the ratio obtained for Ax2. Bars represent means and SD of five independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ (two tailed t-test).

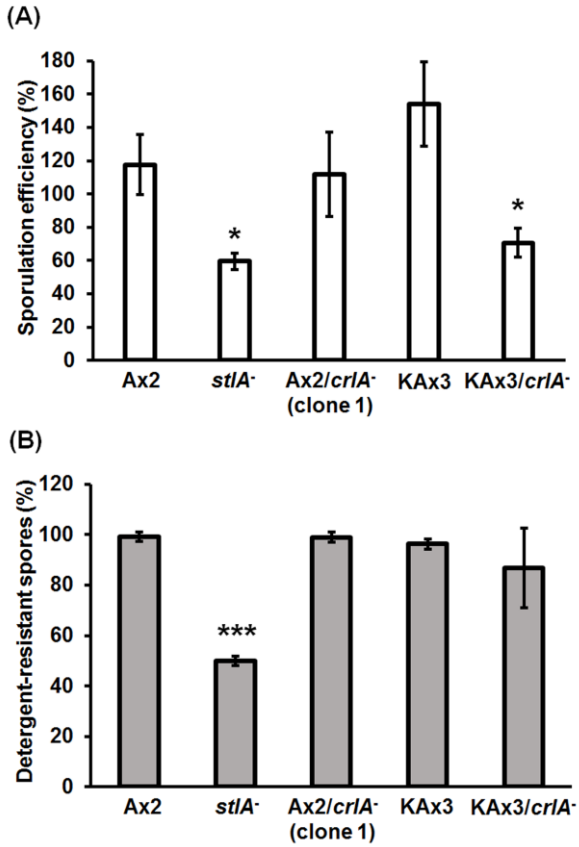

Figure 4. Comparison of spore formation in Ax2/*crlA*$^-$ and KAx3/*crlA*$^-$ strain.

Fixed cell numbers were developed into fruiting bodies on nitrocellulose filters supported by well-buffered agar. The spores were collected and counted after 2 days, then 'sporulation efficiency' (A) and 'detergent-resistant spores' (B) were calculated. Sporulation efficiency was calculated by dividing the number of spores before detergent treatment by the total number of cells plated initially. The ratio of detergent-resistant spores was calculated as described before (see legend of Fig. 3). Bars represent means and SD of 3 experiments, performed in duplicate. *$p < 0.05$, ***$p < 0.001$ (two tailed t-test, vs Ax2).

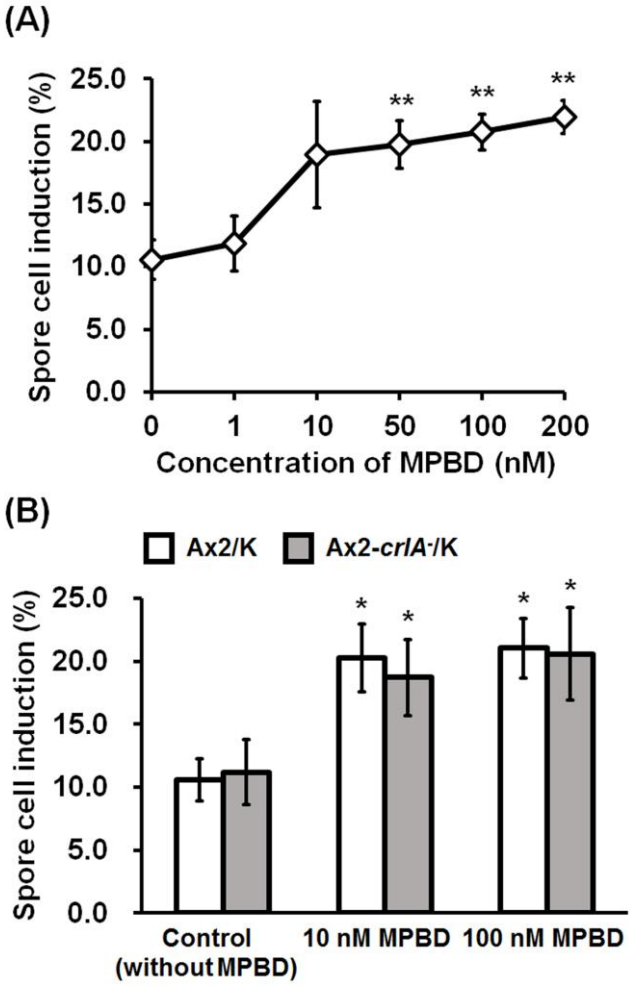

Figure 5. Response to MPBD by Ax2 and Ax2/*crlA*$^-$ strains using sporogenous assay.

(A) Concentration dependence of MPBD induction of spore maturation. Ax2 cells overexpressing *pkaC* (Ax2/K) were incubated in spore salts with 5 mM cAMP at 2×10^3 cells/cm^2. Various concentrations of MPBD indicated were added to the cells after 24 hours, and then spores were counted 2 hours later under the

microscope. Each experiment was performed in duplicate and repeated three times. Bars indicate SD of the three experiments (n=3). ** $p < 0.01$ (two tailed t-test, vs control; 0 nM MPBD). (B) Spore induction by MPBD in Ax2/K and Ax2-*crlA*$^-$/K cells. MPBD was added to the developed cells at concentrations of 10 nM or 100 nM. Spores were scored 2 hours after MPBD addition. Each experiment was performed in duplicate and repeated three times. Bars indicate SD of the three experiments (n=3). * $p < 0.05$ (two tailed t-test, vs Ax2 without MPBD).