J. Phycol. **51,** 247–254 (2015)
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DOI: 10.1111/jpy.12278

ATP AND RELATED PURINES STIMULATE MOTILITY, SPATIAL CONGREGATION, AND COALESCENCE IN RED ALGAL SPORES[1]

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Adenosine 5′-triphosphate (ATP) is a versatile extracellular signal along the tree of life, whereas cAMP plays a major role in vertebrates as an intracellular messenger for hormones, transmitters, tastants, and odorants. Since red algal spore coalescence may be considered analogous to the congregation process of social amoeba, which is stimulated by cAMP, we ascertained whether exogenous applications of ATP, cAMP, adenine, or adenosine modified spore survival and motility, spore settlement and coalescence. Concentration-response studies were performed with carpospores of *Mazzaella laminarioides* (Gigartinales), incubated with and without added purines. Stirring of algal blades released ADP/ATP to the cell media in a time-dependent manner. 10–300 μM ATP significantly increased spore survival; however, 1,500 μM ATP, cAMP or adenine induced 100% mortality within less than 24 h; the exception was adenosine, which up to 3,000 μM, did not alter spore survival. ATP exposure elicited spore movement with speeds of 2.2–2.5 $\mu m \cdot s^{-1}$. 14 d after 1,000 μM ATP addition, spore abundance in the central zone of the plaques was increased 2.7-fold as compared with parallel controls. Likewise, 1–10 μM cAMP or 30–100 μM adenine also increased central zone spore abundance, albeit these purines were less efficacious than ATP; adenosine up to 3,000 μM did not influence settlement. Moreover, 1,000 μM ATP markedly accelerated coalescence, the other purines caused a variable effect. We conclude that exogenous cAMP, adenine, but particularly ATP, markedly influence red algal spore physiology; effects are compatible with the expression of one or more membrane purinoceptor(s), discarding adenosine receptor participation.

Key index words: **ATP and related purines; coalescence; red algal spores; spatial congregation; spore motility; spore settlement**

Purines, such as ADP/ATP (adenosine di or tri phosphate), UDP, or UTP (uridine di or triphosphate) are recognized as ubiquitous extracellular signals from unicellular organisms to mammals (Burnstock 2007, Burnstock and Verkhratsky 2009). It is now well accepted that ADP/ATP are relevant physiological communication chemicals that play roles in diverse physiological and/or pathological events in animals; little is known whether these molecules also participate in plant signaling. The physiological effects of these purines and pyrimidines are mediated by a set of P2 extracellular membrane receptors, which include 8 clones of P2Y receptors, all coupled to protein G and 7 clones of P2X ionic channels almost exclusively activated by ATP and related adenine triphosphates in the cell surface (Burnstock 2007, Coddou et al. 2011). Adenosine is also a physiologically relevant mammalian signal, which interacts with a set of 4 separate plasma membrane receptors also coupled to G proteins as the P2Y receptors (Burnstock 2007). In the extracellular space, adenosine derives from ATP metabolism through ectoATPase hydrolysis (Navarrete et al. 2014). Within the past 5 years, adenine receptors, likewise coupled to G proteins, were described (Von Kugelgen et al. 2008); their pharmacological characterization and physiological role is slowly emerging. At present, few drugs are available to identify and characterize the purine receptor and their likely subtypes.

P2X receptors can be found in all vertebrate species and in many marine invertebrates (see phylogenic tree presented by Fountain and Burnstock 2009). Notwithstanding the well-established relevance of purines as cellular signals in vertebrates and invertebrates, these signals have been scarcely studied in unicellular or multicellular algae with the exception of the green algae, *Ostreococcus tauri* (Fountain et al. 2008). This species is one of the smallest unicellular eukaryotes known, a free-living

[1]Received 22 October 2014. Accepted 6 December 2014.
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Editorial Responsibility: C. Amsler (Associate Editor)

organism with a single chloroplast and mitochondrion. An ATP-gated P2X-like receptor was cloned from this cell which shares ~28% identity with human P2X receptors and ~23% with a receptor cloned from the unicellular social amoeba *Dictyostelium discoideum* (Fountain et al. 2007, 2008, Fountain and Burnstock 2009). The ATP affinity for this primitive ATP-activated P2X receptor is in the high micromolar range (Fountain et al. 2008), almost 100-fold less sensitive to ATP that equivalent mammalian receptors (Coddou et al. 2011). Little is known as yet about the physiology of purinergic signaling in algae, since the receptor seems to be restricted to intracellular compartments as reported for the *D. discoideum* ATP receptors (Fountain et al. 2007, Ludlow et al. 2008, 2009). In vertebrates, adenosine cyclic 3′,5′ monophosphate (cAMP) is derived from ATP by the action of membrane bound adenylyl cyclases; this particular purine plays a major role as an intracellular messenger for a variety of signals from hormones and pheromones to tastants and odorants. cAMP targets intracellular protein kinase A and related kinases, and several regulatory proteins. In the unicellular Myxomycota such as *D. discoideum*, cAMP plays a role as an extracellular signal related to cell movement and cell aggregation of these social amoebas (Kessin and van Looheren Campagne 1992). However, in vertebrates no extracellular action of cAMP is reported since this purine is confined exclusively as an intracellular messenger (Nelson and Cox 2008).

Based on the available physiological findings, we wondered whether cAMP, ATP and/or related purines play a role in macroalgal signaling, facilitating spore aggregation and coalescence. Since red algal spore somatic fusion may somehow be considered analogous to the congregation processes of social amoeba, we thought that purines may act as chemotactic agents as first described for slime molds by Bonner (1947). cAMP was identified as the Myxomycota chemotactic signal (Kessin and van Looheren Campagne 1992) 45 years thereafter the original Bonner evidence. Moreover, since purines in general, and ATP in particular, are ancestral and widespread extracellular cell messengers from unicellular to vertebrates and mammals (Burnstock and Verkhratsky 2009, Fountain 2013), we hypothesized that purines in general may act as extracellular signals, to stimulate movement, spatial congregation and coalescence of carpospores of red algae. To examine this proposal, purine concentration-response experiments were performed evaluating mortality and germination 48 h after spore recruitment in media with and without exogenously added purines. In addition, spore abundance, growth and coalescence of individuals at various distances from the spore seeding spots were also measured 14 d after spore seeding. Experimental results were completed with microscopic observations to visualize spore motility and calculate spore movement speeds.

MATERIALS AND METHODS

Fertile thalli from the middle intertidal seaweed *Mazzaella laminarioides* (Bory) Fredericq were collected between March 2012 and January 2013 in Maitencillo, central Chile (32°30′ S; 71°29′ W). The intertidal habitats and the collection and transportation methods of fertile blades to the experimental laboratory in Santiago have been extensively described by Santelices and Alvarado (2008) and Santelices et al. (2011).

Spore collection and ATP release from fertile blades. In the laboratory, the fertile blades were quickly washed under running tap water, then brushed with filtered (0.22 μm) seawater, dried with paper towel and maintained for 60–90 min under laminar air-flow for desiccation. In order to measure ATP released from fertile blades three glass containers with 200 mL filtered seawater (0.22 μm) and 16 g of blades were stirred in an orbital shaker (SO1 orbital shaker, Stuart Scientific, Stone, Staffordshire, UK) at 100 rpm for 90 min. Three other glass containers with filtered seawater were stirred in parallel. In both treatments 0.2 mL samples were taken at 5, 30, and 60 min stir to determine the nucleotides present, and the number of spores released.

Spore release, accumulation, and planting. To stimulate carpospore release, blades were washed, dried and desiccated, for 2 h as above. Desiccated blades were then sectioned into 3–5 cm long fragments and placed for 1–2 h in 15 cm diameter petri dishes filled with 30 mL seawater at 5°C. Released spores were removed by a thin glass pipette and accumulated within a 100 mL glass container, with 50 mL of filtered seawater and maintained on top of a mechanical rotator at 50 rpm. The spore density was measured in a Neubauer chamber (Boeco, Hamburg, Germany), Densities of 15,000–17,000 spores. in a 0.5 mL of spore mix. were placed in a 6 cm diameter, 1 cm deep rounded, plastic dish (Isolab, Wertheim, Germany) containing 9.5 mL of filtered seawater and the experimental concentration of ATP being tested, with a 10 mL total volume.

Three zones (central, intermediate, and periphery) were distinguished in each plastic dish. The central zone corresponded to the central 1.44 cm of the dish (diameter) and encompassed a total surface of 1.62 cm^2. The peripheral zone was a 1.4 cm wide band and a total surface of 20.23 cm^2, while the intermediate zone also was a 0.88 cm wide band with a total surface of 6.4 cm^2. Spores were carefully placed in the middle point of the peripheral zone, all around the dish; plates were incubated, without movement, for 48 h.

Spore ATP metabolism. To assess whether spores degrade or transport intracellularly ATP from the incubation media, 6 cm diameter plaques were seeded with 1,600 spores · mL^{-1} with known ATP concentrations varying from 1 to 1,000 μM in a final 10.5 mL volume. At the end of the incubation period (1 and 48 h) a 200 μL sample was retrieved to analyze residual ATP in the sea water; the procedure to separate, identify and quantify ATP, and its eventual metabolites, was done in a HPLC equipment (LaChrom Elite L2130; Merck-Hitachi, Minato-ku, Tokyo, Japan) as reported by Buvinic et al. (2007). The experiment was replicated in 10 times. Results are expressed as a % of the mean ± standard error of the mean (SEM) of the ATP retrieved in the aliquot samples attained 1 or 48 h after spore incubation.

Application of ATP to the spore cultures and analytical nucleotides quantification. A stock solution of 10 mM ATP (Sigma-Aldrich, St. Louis, MO, USA) was prepared immediately before each experiment. ATP was diluted in seawater; 9.5 mL of this solution were applied directly to the plastic dishes in dilutions such that the final nucleotide concentration in each dish ranged between 0 (control) and 10,000 μM (10 mM).

Concentrations used were 0, 1, 10, 30, 100, 300, 1,000, 3,000, and 10,000 μM ATP, including an additional 0.5 mL added with the spores.

To estimate the concentration of ATP in the dishes before and 48 h after sporeling seeding, a 100 μL sample was extracted and assayed to determine the nucleotide content. The difference between the ATP applied and that found in the dishes after 48 h may account for ATP degradation and/or uptake via spore nucleotidases and/or transporters or channels.

To assess analytically the concentration of ATP and related purines present in the petri dishes at any time, the nucleotide containing samples were chemically derived using 2-chloroacetaldehyde to form the corresponding etheno fluorescent ATP derivatives. The procedure to separate, identify and quantify ATP and eventually its metabolites followed Buvinic et al. (2007).

A total of 5 plaques with spores were used as replica for each of the 7 ATP concentrations examined. They were incubated for 48 h without movement and under controlled temperature (14°C ± 2°C), photon flux density (30 ± 10 μmol photons · m^{-2} · s^{-1}) and photoperiod (12 h of daily light). After 48 h of incubation, spores were counted and the culture medium changed to SFC culture medium (Correa and McLachlan 1991), renewed every 3 d. Spores were then incubated under the same conditions specified above, measuring spore abundance, sporeling growth and frequency of coalescence after 14 d of age.

Germination, growth, and coalescence. The germination and mortality effects of the multiple exogenous ATP, cAMP, adenosine, or adenine concentrations examined were measured 48 h after spore seeding using 10 randomly selected sampling points marked at the reverse bottom of each capsule. The position of each sampling locus was determined using a numbered grid, drawing the intercepts from a table of random digits. Number of alive and dead spores found in a total surface area of 3.5 mm^2 was counted at each selection point of replicate capsules using an inverted microscope (TMS; Nikon Corp., Tokyo, Japan). Total sporeling abundance at each of the three capsule zones (periphery, intermediate and central) was measured 7 and 14 d after spore seeding, though results are reported with data obtained mainly after 14 d. The position of each sampling point was determined using a grid marked at the bottom of each capsule; photographs were obtained using a stereomicroscope (SMZ-10A; Nikon Corp.) within a square frame of 1 mm^2. Four replicate samples were photographed in randomly selected points in each of the 3 zones distinguished within each replicate capsule. The image of each field was captured using a digital camera (DsFil; Nikon Corp.) and the program Nis Element 3.0 was used to measure disk size of each sporeling and number of spores coalescing after 7 and 14 d of incubation. Diameter of individual germlings was also measured using the Image Pro-Plus v4.5 (Media Cybernetics, Silver Spring, MD, USA).

Microscopic observations. Experimental results were completed with direct microscopic observations. One or two drops of stock solution of ATP at different concentrations were added to one side of a glass slide containing a spore solution. The slide was maintained under low light intensity and humidity, enough to see the changes in motility, shape and response of the spores. Observations were done with a microscope (Optiphot II; Nikon Corp.) with a digital camera Nikon DsFil and the software program NisElement 3.0. Video microscopy was used to record the time course of spore motility. Spores were examined microscopically in a drop of ATP to at a final concentration of 1,000 μM ATP. Pictures were taken every few seconds over a 2 min lapse. As markers, non-motile spores were used. In parallel, spores were fixed in plastic Specimen Embedding Capsules (Beem) with glutaraldehyde and paraformaldehyde after 2, 10, 24, and 48 h ATP treatment. 500 nm sections were obtained for microscopic observations using toluidine blue. These observations were replicated six times.

Statistical analysis. Data on the percent of spore survival, spore abundance at 48 h, 7 and 14 d of experiments, disc area and number of coalescing spores were analyzed using several statistical tests (Sokal and Rohlf 1981). ANOVA followed by Dunnett's Multiple Comparison Test (Graph Pad Programs, San Diego, CA, USA) to compare multiple observations against a common control (Graph Pad Programs). Statistical significance was set in all cases to be $P < 0.05$.

RESULTS

Mild shaking of red algal fertile blades elicited ATP release. Slight stirring of fresh algal blades elicited a linear increase in basal seawater ADP and ATP, however, no adenosine 5′ mono phosphate (AMP) nor adenosine were found in the same samples (Fig. 1). Following 60 min, the basal seawater extracellular ATP rose significantly 9.9-fold, from 1.8 ± 0.4 ($n = 11$) to 17.8 ± 2.7 pmol · mL^{-1} (Student's t-test; $t_{12} = 10.24$, $P < 0.0001$), suggesting that algae react to this mechanical stimuli, releasing ATP and ADP to the extracellular media. Controls, filtered or non-filtered seawater emitted similar basal fluorescence values, allowing us to propose that either seawater produces a background fluorescence, which likely is due to a non-specific effect, or that seawater contains purine traces. The rise in extracellular purines was paralleled to an increased spore population in the cell media; an hour after stirring, the spores augmented from 0 to 5,000 mL; 90 min after stirring, 10,000 spores · mL^{-1} were detected while no spores were observed in the seawater controls.

Spore ATP metabolism. Spores failed to modify significantly the concentration of exogenous ATP added to seawater during 1 or 48 h (Table 1). Even

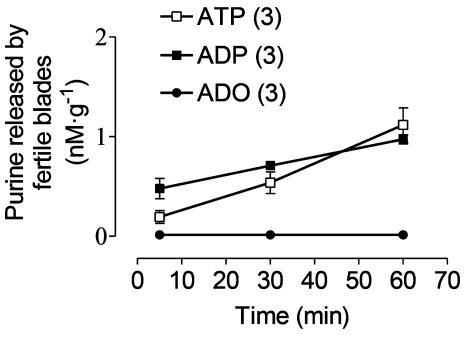

FIG 1. Release of ATP and ADP to the seawater from red algal blades by gently stirring incubation media Symbols represent mean values of the purines examined (nM · g^{-1} fertile blades used); bars are standard errors of the mean. Numbers in parenthesis indicate independent purine determinations. ADO stands for adenosine; ADO and AMP determinations were 0 and did not show variations (AMP data not shown for clarity).

TABLE 1. Lack of ATP metabolism by spores incubated with the nucleotide for 1 and 48 h. Results derived from 10 observations obtained from different spore collections.

	Retrieval of ATP in the spore media	
Initial ATP concentration (μM)	1 h incubation	48 h incubation
1	94.3 ± 2.6	94.3 ± 3.0
10	95.6 ± 4.5	116.4 ± 2.6
30	94.0 ± 10.9	124.9 ± 7.7
100	115.8 ± 3.7	115.2 ± 10.6
300	91.4 ± 13.3	97.3 ± 9.6
1,000	110.4 ± 7.5	103.6 ± 3.2

Values are expressed a % of initial concentration, mean values ± SEM.

though the nucleotide concentrations ranged in 3 orders of magnitude, no significant dependent metabolism change was evidenced. This result allows us to infer that under our experimental conditions ATP is neither significantly metabolized nor transported intracellularly by the spores.

Application of ATP and related purines to the cultures; spore concentration-response survival studies. Only ATP, in concentrations ranging from 30 to 500 μM consistently elicited an 10%–17% increase in spore survival ($F_{4,157} = 5.266$, $P < 0.0005$), reducing correspondingly the spore mortality ($F_{4,159} = 5.238$, $P < 0.0005$; Fig. 2). While 1,000 μM ATP essentially did not affect spore germination, increasing the concentration to 1,500, 2,000 or 3,000 μM elicited, within less than 24 h, 100% mortality (Fig. 2). In contrast, the concentration-response studies for cAMP, shows that 0.1 μM decrease the germination (ANOVA between 0.1 to 300 μM was $F_{6,156} = 3.552$, $P < 0.0025$) Adenine 1–100 μM, did not modify the germination, but concentration above 1,000 μM reduced germination. Adenine was more potent than ATP or cAMP to induce mortality; 300–2,000 μM adenine reduced germination ($F_{2,90} = 108$, $P < 0.0001$), an effect paralleled by a corresponding increase in mortality (Fig. 2). Interestingly, adenosine, did not influence spore survival up to 3,000 μM ($F_{6,126} = 1.372$, ns), an indication that the nucleoside is innocuous; revealing clear structural purine requirements for the toxic effect in spores.

Spore settling and movement. To assess whether the application of exogenous purines modify spore distribution in the seeding plaques, cells were seeded in the central portion of the periphery ring as typified in the diagram presented in Figure 3; 14 d thereafter, the abundance of individuals growing in the 3 pre-assigned dish zones (central, C; intermediate, I; and periphery, P) was examined. In control experiments, the total spores found in the 3 zones were not the same (see columns in Fig. 3) revealing an almost 10-fold spontaneous spore migration toward zone C. At day 14, the number of spores was larger in P, where spores were seeded, decreased in

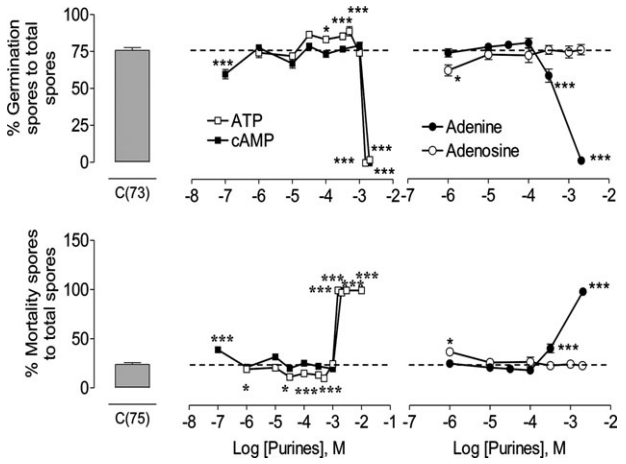

FIG. 2. Effect of ATP and related purines on germination and mortality of carpospores of *Mazzaella laminarioides*. Columns represent the averaged mean values of spore germination/mortality in the absence of exogenous purine addition ($n = 73$ for germination, while $n = 75$ for mortality protocols); symbols represent mean, average values; bars, standard error. The left panel shows ATP (open squares) or cAMP (closed squares) effects. Right panel shows adenine (close circles) and adenosine (open circles). *, $P < 0.05$; ***, $P < 0.001$ as compared to the controls spores seeded simultaneously, except for the addition of purines (Dunnett's Multiple Comparison Test). Dotted line represents the mean control value to which the data should be compared.

abundance toward the I and C zones. While no effects in spore distribution were observed in spores treated with 1–30 μM, 100 μM ATP increased spores in the center ($F_{5,34} = 5.89$, $P < 0.0005$, and a dramatic effect was attained with 1,000 μM ATP since we consistently observed a 3- to 4-fold increase in spore abundance in region C, 14 d after planting. Moreover, as anticipated, the increased in spore abundance in C was paralleled by a significant decrease of individuals in P between 1 and 100 μM ($F_{6.43} = 6.637$, $P < 0.0001$). No significant changes were observed in the intermediate zone. Similar effects were also observed 7 d after the 1,000 μM ATP treatment (data not shown).

While adenosine did not cause significant changes in the distribution of individuals in the 2 zones of the plates, except for a minor decrease in P ($F_{6,38} = 5.871$, $P < 0.0002$), with no correspondence in the I or C zones, 1–1,000 μM cAMP showed a significant yet minor increase in the number of individuals in C ($F_{6,38} = 5.699$, $P < 0.0003$), with no correlations with the other plate zones. Adenine showed a different profile, as we observed no change in the number of individuals in P, paralleled by a corresponding significant increase in the I ($F_{5,54} = 3.228$, $P < 0.0127$ and C ($F_{5,34} = 8.793$, $P < 0.0001$) areas. While qualitatively the effects of adenine were similar to ATP, adenine effects were attained with lower concentrations, albeit the magnitude of the effect was not as marked as with ATP.

In addition, we carefully examined whether the purines altered the total number of spores distrib-

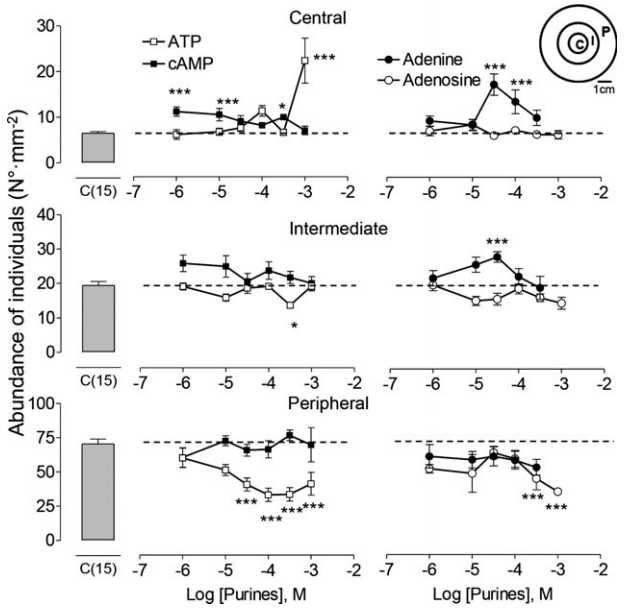

FIG. 3. Effects of ATP, cAMP, adenosine and adenine on spore distribution in seeding plates. The plaque zones were labeled as peripheral (P), intermediate (I) or central (C) as shown schematically in the accompanying diagram (upper right part). Controls ($n = 15$) show the distribution of spore abundance in dishes seeded simultaneously except for purine addition. Columns represent the averaged mean values of spore abundance in 15 control plates; symbols represent mean values. Bars correspond to the standard error. The left panel shows ATP (open squares) or cAMP (closed squares) effects. Right panel shows adenine (close circles) and adenosine (open circles). *, $P < 0.05$; ***, $P < 0.001$ as compared to the respective controls (Dunnett's Multiple Comparison Test). Fine dotted line represents the mean control value to which the data were compared.

uted in the 3 dish zones. Nucleotides did not modify, within statistical variations, the total number of spores in the plaques (data not shown), allowing us to conclude that nucleotides influence spore distribution by enhancing spore distribution toward the C dish zone. Therefore, these purines, with the exception of adenosine, directly affected spore motion accounting for the individual distribution observed. In addition, the differences in purine profiles suggest structural requisites compatible with the putative activation of one or more of the purine receptors; excluding functional spore adenosine receptors.

Microscopic observations. To directly examine whether ATP influenced spore motility, microscopic observations confirmed that 1,000 μM ATP-induced motility as soon as 2 h after spore release (Fig. 4). Using sequential timed frames, spore speeds were calculated in a dozen individuals; speeds ranged from 2.2 to 2.5 $\mu m \cdot s^{-1}$. Moreover, careful microscopic observations showed that motility was not continuous; it appeared to involve cytoplasmic rotation and/or cell gliding, consistent with the absence of cell flagella. In addition, we consistently observed that spores apparently avoided obstacles, such as

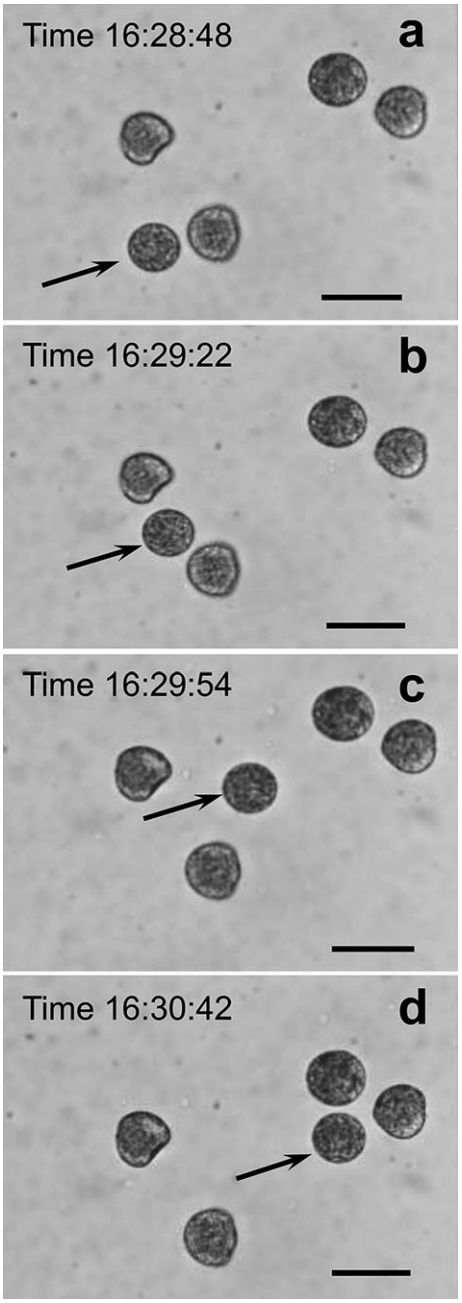

FIG. 4. Direct evidence of spore motility and capacity to avoid obstacles and speed. Arrows indicate a migrating spore; picture calibration scale represents 25 μm.

dead spores used as space markers. A representative 2-min time frame set of pictures is presented in Figure 4. Control spores not exposed to ATP did not evidence movement within the same observation time frame, an indication that ATP triggers spore displacements.

Spore growth and coalescence. Quantification done 14 d after seeding indicated that spore incubation with 1,000 μM ATP increased the abundance of coalescing individuals, an effect particularly marked in

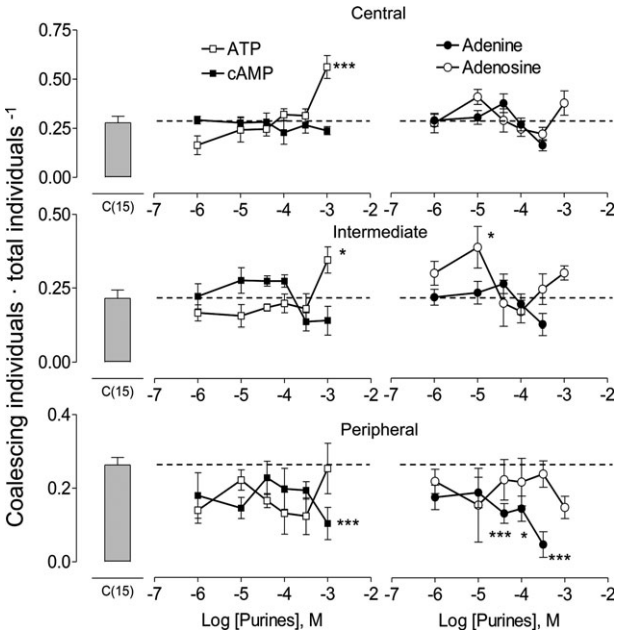

FIG. 5. Effects of ATP on spore coalescence. Columns indicate the averaged values of 15 control experiments in the absence of purine addition; symbols represent mean values. Bars correspond to standard errors. The left panel shows ATP (open squares) or cAMP (closed squares) effects. Right panel shows adenine (close circles) and adenosine (open circles). *, $P < 0.05$; ***, $P < 0.001$ as compared to the controls using Dunnett's Multiple Comparison Test. Dotted line represents the mean control value.

the C and I dish zones (Fig. 5). Lower concentration of ATP (1–300 μM) did not modify coalescence in C and I, but decreased coalescence in P ($F_{5,36} = 3.882$, $P < 0.0069$). Microscopic observations indicated that 1,000 μM ATP stimulated spore coalescence within 10 h or less. In addition, we observed that 24 h after ATP exposure, the degree of coalescence was similar to that attained in control spores 48 h after seeding; this observation suggests that ATP significantly enhanced spore coalescence, and that nucleotide accelerates a cell response that will occur naturally at least 24 h later. Additions of cAMP, adenine or adenosine did not modify coalescence as markedly and as consistently as ATP in C and I. cAMP ($F_{6,38} = 2.476$, $P < 0.0404$) and adenine ($F_{5,54} = 4.65$, $P < 0.0013$) significantly reduced coalescence in a concentration-dependent pattern in the P zone.

DISCUSSION

Present results consistently indicated that exogenous ATP applications, increased motility, aggregation and coalescence in recently released carpospores of *M. laminarioides*, a finding in a way reminiscent to the *D. discoideum* biology; social amoeba use a gradient of cAMP as a single cell chemoattractant until the slime mold is formed (Kessin and van Looheren Campagne 1992).

Other exogenous purines such as cAMP or adenine also mimiced the action of ATP on algal spore aggregation, an observation that is of physiological relevance, suggesting that likely endogenous purines may induce similar spore responses. While cAMP or adenine were more potent than ATP to influence spore distribution, the magnitude of the responses was less robust than the ATP-induced response. On the basis of structure activity relationships documented, we infer, on an interpretative basis, that the effects described may be mediated by one or more ATP receptors as those reported in vertebrates or in the green algae, *O. tauri* (Burnstock 2007, Fountain et al. 2008, Coddou et al. 2011). The finding that adenosine was inactive highlights the notion of selectivity within the purines examined, and is consistent with the concept of purinoceptor signaling. The result that relatively high concentrations of ATP were required to induce motility, aggregation and coalescence in red algae spores is consistent with the finding that the green algae P2X receptor cloned has micromolar affinity for ATP (Fountain et al. 2008), highlighting the observation that ancient ATP receptors, such as found in unicellular organisms, have lower ligand affinity than counterpart vertebrate receptors (Coddou et al. 2011, Navarrete et al. 2014).

We systematically observed that 100–1,000 μM ATP not only stimulated spore motility and aggregation but facilitated settlement, coalescence and cell wall formation. Although a number of abiotic factors are known to contribute to spore settlement (see reviews by Santelices 1990, Fletcher and Callow 1992, Amsler 2008, 2012), this is the first report showing that exogenous ATP modified spore and strongly influenced the displacement of spores within the dish zones. Purines may act directly as chemotactic agents or indirectly through cell reactions elicited following extracellular purine receptor signaling. Therefore, we suggest that purines should be included within the endogenous chemicals that contribute to spore survival, motility and coalescence.

The present results unequivocally showed that purines have a dual effect on spore survival. We described an optimal concentration of ATP and related purines that increased motility, germination and coalescence, while increasing purine concentrations over 1,500 μM caused 100% spore death. This bell-shaped concentration-dependence indicates that the regulation of extracellular ATP concentration is critical for spore growth and survival. This is a puzzling observation in light of the observation of no significant ATP metabolism by the spores within an ample range of purine concentrations. As to how the extracellular ATP concentration is regulated to avoid lethal effects remains unknown. Notwithstanding, if one assumes that fertile blade movement results in ADP/ATP secretion, it is possible to infer that in the algal microenvironment both physical and chemical

signals contribute to spore settlement. A chemical gradient, such as the one proposed to occur with purines, might be relevant to increase settlement and coalescence in low dispersal spores. Dilution factors in the real marine setting may reduce such a capacity in long-range distances. We repeatedly observed that ATP speeds cell coalescence by ~24 h, a process that occurs naturally in the spores, giving rise to the notion that the purine accelerates a complex cellular response that involves, among other processes, cell mitosis and important membrane extensions in the new germlings.

Cell motility stimulated by extracellular ATP plays an important role in spore aggregation. The present results confirm red algal spore motility which, by now, has been described for several species (Pickett-Heaps and West 1998, Zucarello et al. 2000, Pickett-Heaps et al. 2001, West et al. 2005, 2007a,b, 2008, Ackland et al. 2006, 2007, Wilson et al. 2006, Scott et al. 2008), reaching speeds of 2.2–2.5 $\mu m \cdot s^{-1}$, values similar to those found in our measurements. In the case of *Porphyra pulchella*, spores exhibited gliding and, more frequently amoeboid movements (Ackland et al. 2006), as we also observed. In this species, motility appears to be related to cytoskeleton rearrangements as the use of actin and myosin inhibitors disrupted F-actin networks and reversed pseudopodia activity (Ackland et al. 2007). The force for pseudopodial protrusion seems dependent on actin and myosin interactions. In our experiments ATP signaling, directly or indirectly may be related to cytoskeletal rearrangements, facilitating cell motility.

The few studies describing ATP effects on algae (Palenik et al. 2007) have not examined the mechanism(s) that substantiate the described effects. The physiology of purine signaling in macroalgal cells has not been addressed nor established whether endogenous purines ultimately involved in red algal spore signaling account for the biochemical mediators involved in the aggregation, settlement or coalescence of spores. In spite of this, we propose that purine receptor activation is essential to account for the observed effects. As to how this receptor is linked to define intracellular signaling cascades that accelerate these developments remains unknown and needs further analysis. Nevertheless, the present results allow us to exclude adenosine as a relevant signal in spore responses, validating the concept of ligand receptor selectivity.

Altogether, these novel ATP and purine-mediated responses in red algal spores open up a yet unexplored area of cell physiology in macroalgae, highlighting the role of purines, including adenine, as primitive extracellular cell signals. On the basis of these results, we hypothesize that one or a few spores, by an as yet unknown mechanism, release extracellular ATP, as we demonstrated that gentle physical movement of algal blades, results in release of ATP to the extracellular medium. We infer that the secreted ATP, or a related endogenous purine, may act as a chemotactic messenger to initiate cell aggregation in the central zone of the seeding plates. This signal is likely detected by neighboring spores inducing congregation toward the central dish zone, as inferred from the aggregation experiments. As to why aggregation occurs preferentially in this portion of the plaque, remains unknown. Additional experiments and observations are needed to evaluate whether the red algal spore aggregation is similar in diffusion dynamics and involves cell migration patterns comparable to those described for the social amoeba (Kessin and van Looheren Campagne 1992). As well as to elucidate how extracellular nucleotides could facilitate chemotaxis. We therefore propose that red algal spores likely express one or more ATP receptor(s) proteins in the cell membrane to sense these signal molecules and start intracellular signaling cascades consonant with the physiological results observed. Regarding cAMP, while vertebrates lack this cell surface receptor site, since cAMP is essentially an intracellular nucleotide (Nelson and Cox 2008), perhaps these red algal spores conserve a selective cell membrane receptor for this nucleotide as reported for social amoeba.

In summary, although we do not understand the physiological mechanism(s) of the purines-induced spore responses, particularly its effect regulating cell movement, the present results suggest the opportunity of using ATP or related purines to stimulate motility, aggregation and attachment of red algal spores. We deem that a better understanding of the processes and the nature of the chemicals that regulate movement, settlement and coalescence in spores may allow significant improvements in the recruitment and cultivation techniques presently used to produce economically important spore-propagated algae and a better understanding of the role that purine, such as ATP, cAMP or adenine play in intra and extracellular cell communication in red algal spores.

The authors appreciate data and statistical analysis by Dr. Marcelo Bobadilla. This study was partially supported by grants FONDECYT 1120129 (to BS) and 1141132 (to JPGHT).

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