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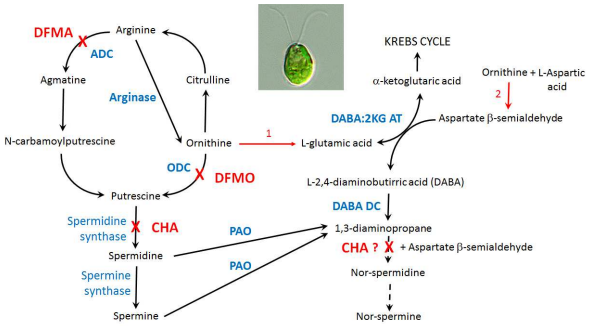

**Title:**

# Effect of ornithine decarboxylase and norspermidine in modulating cell division in the green alga *Chlamydomonas reinhardtii*

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**Abstract**

The extensive genetic resources of *Chlamydomonas* has led to its widespread use as a model system for understanding fundamental processes in plant cells, including rates of cell division potentially modulated through polyamines. Putrescine was the major polyamine in both free (88%) and membrane-bound fractions (93%) while norspermidine was the next most abundant in these fractions accounting for 11% and 6%, respectively. Low levels of diaminopropane, spermidine and spermine were also observed although no cadaverine or norspermine were detected. Ornithine decarboxylase (ODC, EC 4.1.1.17) activity was almost five times higher than arginine decarboxylase (ADC, EC 4.1.1.19) and is the major route of putrescine synthesis. The fluoride analogue of ornithine (α-DFMO) inhibited membrane associated ODC activity whilst simultaneously stimulating cell division in a dose dependent manner. Following exposure to α-DFMO the putrescine content in the cells declined while the norspermidine content increased over two fold. Addition of norspermidine to cultures stimulated cell division mimicking the effects observed using DFMO and also reversed the inhibitory effects of cyclohexylamine on growth. The results reveal that ODC is the major route to polyamine formation in the *Chlamydomonas* CC-406 cell-wall mutant, in contrast to the preferential ADC route reported for *Chlorella vulgaris,* suggesting that significant species differences exist in biosynthetic pathways which modulate endogenous polyamine levels in green algae.

**Keywords**

Polyamines, *Chlamydomonas*, ornithine decarboxylase, arginine decarboxylase, α-difluoromethylornithine (DFMO), α−difluoromethylarginine (DFMA).

## 1. Introduction

The unicellular green alga, *Chlamydomonas* has proved a useful system in which to study aspects of plant cell division, largely resulting from its ease of cultivation and the ability to manipulate its growth in culture. The occurrence of polyamines and their biosynthetic enzymes is associated with high growth rates and active cell division in plant, bacterial and animal systems (Handa and Mattoo, 2010; Kusano et al., 2008). Among the numerous functions attributed to polyamines during cell cycle are the sequestering of negative charges on the phosphate groups of nucleic acids and, in particular in *Chlamydomonas*, roles in actin filament formation associated with spindle formation and cytokinesis (Baron and Stasolla, 2008; Lee et al., 1997). The principle polyamines found in plant tissues are the diamine putrescine, the triamine spermidine and the tetra-amines spermine and thermospermine formed by the sequential addition of aminopropyl groups from *S*-adenosylmethionine (Kusano et al., 2008; Takano et al., 2012). It is generally accepted that in animals putrescine is synthesised from ornithine decarboxylase (ODC, EC 4.1.1.17), whereas in plants and bacteria an additional pathway, mediated by arginine decarboxylase (ADC, EC 4.1.1.19), is present (Carbonell and Blazquez, 2009). The relative activities of these two enzymes appear, in part, to be regulated by a variety of factors including abiotic stress (Alcazar et al., 2010; Bagni et al., 2006; Groppa and Benavides, 2008). In eukaryotes, spermidine in particular is essential for cell proliferation and normal cell growth and development. Besides being involved in basic cellular processes, such as ribosome function and mRNA translation, spermidine is also required for modification of a lysine residue in eIF5A to produce the unique amino acid hypusine. Hypusine formation is essential for cell proliferation and growth, at least in yeast and mammalian cells (Wolff and Park, 2015). In addition to common polyamines, in green microalgae, norspermidine, norspermine, thermospermine and homospermine were also detected (Hamana et al., 2004; Hamana and Matsuzaki, 1982; Hamana et al., 2013). Despite significant advances in understanding cell division in *Chlamydomonas* (Theiss et al., 2004; Theiss et al., 2002; Vitova et al., 2011; Vitova et al., 2011), studies have only been undertaken on ODC in this organism and the activity of this enzyme was shown to increase during cell division (Theiss et al., 2002; Voigt et al., 2004). It has been suggested that single-celled green algae have lost the arginine route and are dependent, like other eukaryotes, on putrescine biosynthesis from ornithine (Fuell et al., 2010). However, in *Chlorella*, both ODC and ADC activities were detected although only an increase in ODC activity was observed during logarithmic phase of growth (Cohen et al.,

1983). In *Chalmydomonas reihardtii* two different genes coding for ODC (ODC1: gene ID 5723008; ODC2: gene ID 5724425) and one gene for an arginine/ornithine decarboxylase mostly similar to bacterial decarboxylase (OAD1: gene ID 5721472), have been described and annotated after the complete genome sequencing (Merchant et al., 2007). However, no protein expression data linked with enzymatic activity for these two enzymes, has been reported.

In order to elucidate the role of ODC and ADC in polyamine synthesis and to assess their effects on cell division, we chose a cell wall mutant of *Chlamydomonas reinhardtii* (CC-406 cw 15 $mt^-$). In the wild type cell walls are composed of a multilayered structure consisting of a hydroxyproline-rich glycoprotein framework with galactose, arabinose and mannose being the predominant carbohydrate side chain. Other components include glucosamine, uronic acids and proteins (Blumreisinger et al., 1983; Popper et al., 2011). The mutation of the *C. reinhardtii* (CC-406 cw 15 $mt^-$) strain is described as cell wall deficient, based on the incorrect formation of proteins and extracellular cross linking resulting in minimal amounts of cell walls being produced (Harris, 2001). This mutant offers potential advantages over the wild type since addition of exogenous compounds to the culture medium would be more readily taken up by the cells coupled with the ease of counting the newly formed cells, which readily separate from each other after division, in contrast to the wild type where the daughter cells are retained inside the original cell wall for some time afterwards. Here we report the effects of manipulating the endogenous levels of polyamines and their effects on ODC and ADC activities and how this influences cell division.

## 2. Materials and methods

### *2.1 Materials*

*C. reinhardtii* cell wall mutant strain (CC-406 cw 15 $mt^-$) was obtained from the Chlamydomonas Genetics Center, Duke University, Durham, USA. *C. reinhardtii* wild type (CC-125) obtained from the CCAP (Culture Collection of Algae and Protozoa, Oban, Scotland/UK). Putrescine, spermidine, spermine, norspermidine and norspermine were purchased from Sigma, UK. D,L-[1-$^{14}$C] ornithine (specific activity, 56 mCi $mmol^{-1}$) and L-[U-$^{14}$C] arginine (specific activity, 303 mCi $mmol^{-1}$) were purchased from Amersham, UK).

α-Difluoromethylornithine (DFMO) and α–difluoromethylarginine (DFMA) were kindly supplied by Hoechst Marion Roussel (Ohio, USA).

*C. reinhardtii* (CC-406 cw 15 mt$^-$ and CC-125) were cultivated under axenic conditions in TAP (Tris-Acetate-Phosphate, pH 7.0) medium containing Hutners trace elements in a New Brunswick Scientific orbital shaker with continuous rotary agitation at 12.6 rad $s^{-1}$, at 20 °C under cool white fluorescent lighting with a 12 h photoperiod. At 24h intervals, aliquots were removed from the culture flasks and cell number was determined, in triplicate, using an automated haematology analyser (Coulter, counter S8 80, Coulter Electronics, Luton, UK) where the cells were detected in the platelet window. Cells were cultured in either 24 well titre plates (Nuncleon, Denmark) in 1.8 mL TAP medium or in 500 mL conical flasks in 149 mL TAP medium inoculated with 1 mL stationary phase cells (2.5x $10^7$ cells) containing inhibitors or exogenously supplied polyamines at concentrations ranging from 0.01 to 10 mM alone or in combination. The cells were harvested at 24 h intervals, re-centrifuged and immediately frozen in liquid nitrogen and stored at -80ºC until required.

*2.2. Analysis of endogenous polyamines*

After 3 days of growth in culture media supplemented with polyamine inhibitors, cells were harvested by low speed centrifugation at 29 m $s^{-2}$ for 15 min. The pellet was weighed and re-suspended in fresh media without inhibitors and re-pelleted. Stored samples (in -80 ºC) were defrosted and 200 mg wet weight aliquots were disrupted in a Potter Eljehem homogeniser in 10 vol perchloric acid (PCA, 4% v/v). After 1 h incubation at room temperature, the samples were centrifuged at 20,000 g for 30min at 4 °C. The pellet was re-suspended in the original volume of PCA. Triplicates of this suspension and of the supernatant were hydrolysed with 6 N HCl in flame-sealed vials at 110 °C for 20 h in order to release polyamines from their conjugates. Aliquots (0.2 mL) of supernatant (free polyamines), hydrolysed supernatant (soluble-bound polyamines) and hydrolysed pellet (insoluble-bound polyamines) were dansylated, toluene extracted and separated by HPLC as described previously (Tassoni et al., 2000).

*2.3. ODC and ADC assays*

*Chlamydomonas* cells were homogenised in 4 vols of ice cold assay buffer (100 mM Tris-HCl, pH 8.5 containing 50 μM pyridoxal phosphate) and centrifuged at 26,000g for 30 min at 4 C. Aliquots (0.1mL) of both supernatants and re-suspended pellets were assayed for ODC and ADC activity in the presence of 0.2 μCi [$^{14}$C] ornithine (specific activity, 56 mCi $mmol^{-1}$) and [$^{14}$C] arginine (specific activity, 303 mCi $mmol^{-1}$) respectively by measuring the rate of

decarboxylation i.e. [$^{14}$C] $CO_2$ evolution from the substrates as described previously (Tassoni et al., 2000). To quantify the amount of non-enzymatic decarboxylation, control samples for both ODC and ADC assays were performed and in particular: a) control without any algal extract; b) control by using boiled algal extract. The obtained control levels were subtracted from those of the samples. Protein content was determined using the Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard.

*2.4. Arginase activity*

Arginase activity (EC 3.5.3.1) was assayed by measuring the Arg-dependent production of urea essentially according to (Goldraij and Polacco, 1999). One mL of standard assay medium contained 160 mM L-Arg (adjusted to pH 9.7 with KOH), 33 μM phenyl phosphorodiamidate, an urease inhibitor (Liao and Raines, 1985) and 0.1 to 0.3 mg of extract protein. The reaction mixture was incubated for 30 min at 30°C. Aliquots (400 μL) were removed and the reaction was stopped by adding 1 N $H_2SO_4$ to 600 μL. Urea released was determined colorimetrically and arginase activity was expressed as nmol of urea released/min/mg of protein. Protein was determined by the BCA method (Wiechelman et al., 1988).

*2.5. Statistical analyses*

All the treatments and analyses were performed twice with two biological replicates each. The values are the mean ± SD of four samples in all the experiments. Statistically significant differences between data were analysed using the Student's *t* test ($p<0.05$) (Statistica 6 programme, Statasoft Inc., USA).

## 3. Results

*3.1. Effect of DFMO and DFMA on cell division*

In order to evaluate the potential role of both ODC and ADC in polyamine synthesis, cells were incubated in the presence of the fluoride analogues, DFMO and DFMA, respectively and their growth rates monitored. Cells were incubated with increasing concentrations of DFMO or DFMA ranging from 0.5 mM to 10 mM for up to 120h (stationary phase) (Fig. 1). DFMO stimulated cell division in a dose dependent manner and after 120h incubation cell numbers were increased by 33%, 58% and 78% respectively in the presence of 1, 5 and 10 mM of the inhibitor compared with no fluoride analogue controls (Fig. 1a). In the presence of

DFMA the highest stimulation in cell numbers was observed after 72h incubation for all concentrations tested, again in a dose dependent manner with a maximal 64% increase in cell numbers following exposure to 10 mM of the compound (Fig. 1b). When both inhibitors were added together a similar effect to that with single inhibitor additions was observed (data not shown). Thus it is evident that cell division in *C. reinhardtii* is not arrested by these suicide inhibitors and, on the contrary, cell division is stimulated by their presence.

### *3.2. Effect of DFMO and DFMA on ODC and ADC enzymatic activities*

ODC and ADC activities were determined in supernatant and pellet fractions of cells obtained after incubation of cultures for 72h in either 5 mM DFMO, 5 mM DFMA or 5 mM of both inhibitors in combination. The results (Table 1) reveal that ODC activity, which is about five times higher than ADC activity in controls, both in the supernatant and pellet fractions, is partially inhibited by DFMO in the supernatant (14%) and more strongly inhibited in the pellet fraction (46%). DFMA had no effect on the ODC activity in the supernatant fraction although 23% inhibition was observed for the pellet fraction. This apparent inhibition of ODC activity by DFMA could be accounted for by two principle factors. Either DFMA could be hydrolysed to DFMO by arginase activity and/or the supplied [$^{14}$C]ornithine is metabolised by ornithine transcarbamylase and thereby reducing the concentration of substrate available to undergo decarboxylation by ODC. DFMA, as expected, inhibits ADC activity in both supernatant and pellet fractions (43 and 26% respectively) while DFMO only inhibited ADC activity in the pellet (39%). In the presence of both inhibitors ODC activity was inhibited to a greater extent in the supernatant and to a similar level in the pellet whereas ADC activity was inhibited to a lower level in both preparations compared with DFMA used alone. A likely possible explanation for these observations is degradation, by arginase, of the [$^{14}$C]arginine used to assay ADC activity. This arginase activity could account for the apparent inhibition of ODC in the pellet by converting DFMA to DFMO and could also degrade the arginine substrate used for ADC activity measurements resulting in an apparent ADC inhibition.

### *3.3. Arginase activity*

Arginase hydrolyses arginine to ornithine and urea and the latter was determined colorimetrically. The specific activity of the enzyme was determined to be respectively 65 ± 15 nmoles $min^{-1}$ $mg^{-1}$ protein for the soluble fraction and 185 ± 21 nmoles $min^{-1}$ $mg^{-1}$ protein

for the pellet preparation. The almost three-fold higher arginase activity detected in the latter fraction may, therefore, accounts for the higher inhibition of ODC by DFMA and also of ADC by DFMO in pellet fractions. The higher arginase activity in the pellet agrees with observation in *Arabidopsis*, where again this activity was reported higher in the pellet than the supernatant (Bagni et al., 2006).

*3.4. Endogenous polyamine content in cells incubated with DFMO and/or DFMA*

In control cultures 75% of the total cellular polyamines were detected in the free-state and the remainder were present as soluble-bound forms (Table 2). Only trace levels were detected in the insoluble-bound fraction (<0.1%, data not shown). Putrescine was the major polyamine in both free and covalently-bound fractions accounting for 88 and 93% of the total while nor-spermidine was the next most abundant polyamine with relative abundances of 11% and 6%, respectively. Low levels of diaminopropane, spermidine and spermine were also observed although no cadaverine or norspermine were detectable in any fraction.

Previous papers reported total polyamine levels in wild type species of *C. mowusii* and *C. reinhardtii* by using respectively TLC and HPLC quantification methods (Hamana et al., 2004; Hamana and Matsuzaki, 1982). The total polyamine levels here reported on *C reinhardtii* cell wall mutant, fall within the same range of those previously published for *Chlamydomonas* wild type species, being in particular 2 to 3-fold lower than in *C. mowusii* (Hamana and Matsuzaki, 1982) and 2 to 3-fold higher than in *C. reinhardtii* (Hamana et al., 2004).

After 72h of culture in 5 mM DFMO, the total polyamine content in the CC-406 cells had decreased by some 17± 2% and the relative distribution of free to covalently-bound had also decreased to 61±4%. The relative abundance of putrescine decreased to 75 ± 3 % in the free fraction and to 70±3% in the covalently-bound fraction while an increase in the content of norspermidine was observed up to 25±3% and 29±2% in these fractions, respectively. In comparison, DFMA had little effect on the quantity or on the distribution of free versus bound polyamines. However, the level of bound norspermidine increased significantly from 94 to 153 nmol $gfw^{-1}$ following DFMA treatment. Thus the stimulation of cell division by both DFMO and DFMA (Fig.1) is associated with an increase in norspermidine by either fluoride analogues. Furthermore, in the presence of both inhibitors, the effects were similar to those reported with DFMO alone in which the putrescine content declined and that of norspermidine increased.

*3.5. Effect of exogenous polyamines on cell division*

*C. reinhardtii* cultures were supplemented with exogenous putrescine, spermidine, norspermidine or spermine at final concentrations ranging from 0.1 mM to 10 mM in TAP media in 24 well culture plates and cell numbers determined at regular intervals up to 120h. High concentrations of polyamine application to cultures in the range of 1-10 mM were inhibitory to cell growth in the order spermine>spermidine>norspermidine>putrescine (data not shown). However, tests performed at 0.1 mM revealed that norspermidine stimulated cell division resulting in an almost doubling of cell numbers after 48h incubation (Fig. 2). By contrast, little effect was observed with putrescine and spermidine, whereas spermine inhibited cell division by almost 50%.

*3.6. Effect of cyclohexylamine on cell divison*

Cyclohexylamine (CHA) is an inhibitor of spermidine synthase and may, therefore, influence the pool size of norspermidine in cells. To investigate this, cells were cultured in the presence of 0.1 mM CHA and a range of polyamines (0.1 mM) in order to determine their effects on cell division (Fig. 3). CHA treatment alone reduced cell numbers by 23%, 46% after 24, 48h respectively and between 16-19 % thereafter (72-120h). The polyamine content of the treated cells was also determined. CHA resulted in a higher content of putrescine on both an absolute basis (nmol $gfw^{-1}$) and relative (%) basis (control 83±3 %, CHA 89±3 % $p < 0.05$, n=4) while the content of norspermidine was reduced from 14.0 ±1.0 % to 9.9 ±2.0 % ($p<0.05$, n=4). The content of spermidine was also reduced in CHA treatment by some 46% (or from 2.7 ±0.5 % to 1.3 ±0.5 % of total polyamines, $p<0.05$, n=4). No significant differences were observed in the covalently-bound polyamines in the PCA-soluble fraction and only trace levels of PCA-insoluble polyamines were detected (data not shown). In order to determine whether norspermidine could specifically overcome the metabolic block induced by CHA, cells were incubated with both the inhibitor (0.1 mM) supplemented with either putescine, spermidine, norspermidine or spermine (0.1 mM) (Fig. 3). After 48h of culture, putrescine and spermidine had a slight stimulatory effect of 10 and 22 % on growth whereas norspermidine significantly stimulated culture growth by some 111% compared with the CHA control. By contrast, spermine was slightly inhibitory (3%). After 72h incubation the only polyamine to stimulate culture growth was norspermidine by 22% with all the others being inhibitory in the order spermine, putrescine and spermidine. Indeed, norspermidine was

the only polyamine tested that was able to maintain cell culture growth at the same overall rate as control cells in the absence of inhibitor. Thus, norspermidine is able to reverse the inhibitory effect of CHA on growth.

## 4. Discussion

Both ODC and ADC activities were detected in *Chlamydomonas* with ODC activity being about five times higher. Thus ODC appears to be the major route leading to putrescine formation in this species. By contrast, in *Chlorella vulgaris* (also in the Division *Chlorophyta*), the converse is observed and ADC appears to be the predominant activity being some four-fold higher than ODC (Cohen et al., 1983). Further major differences were observed in the two species response to the suicide inhibitors, α-DFMO and α-DFMA. Both inhibitors stimulated cell division in *C. reinhardtii* whereas in synchronised cultures of *Chlorella*, DFMO inhibited cell division after the first and second cycles, an effect which could be partially reversed by addition of putrescine. By contrast the results here reveal putrescine to be inhibitory to cell division in *C. reinhardtii*. α-DFMO resulted in a lowering of the putrescine content and a marked increase (almost seven-fold) in norspermidine, particularly in the bound fraction. The presence of norspermidine (and norspermine) in aquatic organisms, including thermophilic and halophilic bacteria, arthropods and higher plants is suggested to play possible roles in thermostability or salt tolerance (Hamana and Matsuzaki, 1982). In the present context it is interesting that partial inhibition of putrescine synthesis by α-DFMO should result in increased norspermidine production in the cells suggesting that the suicide inhibitor may also act as an abiotic stress agent.

In bacteria, the complete pathway of norspermidine biosynthesis was recently established; the proposed pathway is enzymatically distinct from that of spermidine (Hobley et al., 2014; Lee et al., 2009). According to the proposed pathway, norspermidine biosynthesis is derived from aspartate β-semialdehyde donating an aminopropyl group to 1,3-diaminopropane (1,3-DAP). Aspartate β-semialdehyde also contributes to the formation of 1,3-DAP itself (via the formation of L-2,4-diaminobutyric acid, DABA) (Lee et al., 2009). An earlier study on the bacterium *Acinetobacter baumannii* demonstrated that 1,3-DAP is formed from L-glutamate and aspartate β-semialdehyde by the action of the enzyme diaminobutyrate:2-ketoglutarate aminotransferase (DABA:2-KG AT, EC 2.6.1.76). DABA is then converted to 1,3-DAP by a DABA decarboxylase (DABA DC, EC 4.1.1.86). Both genes have been cloned and

characterised (Ikai and Yamamoto, 1997). 1,3-DAP can also be synthesised by oxidation of spermidine and spermine by polyamine oxidase (Tavladoraki et al., 2012).

ODC is a highly regulated enzyme (Pegg, 2006) and, according to our data, in *C. reinhardtii* the partial inhibition of this activity with DFMO would generate elevated levels of ornithine which could be converted to L-glutamic acid (Malaisse, 2003) and be channelled in to the Krebs cycle resulting in the production of a-ketoglutaric acid and DABA and/or, if similar to bacterial biosynthetic pathway, could contribute directly to the formation of aspartate β-semialdehyde (Hobley et al., 2014; Lee et al., 2009). These processes would provide substrate for the DABA:2KG AT and could support a route for the synthesis of norspermidine (Fig. 4). We are currently evaluating this possible route of synthesis using radioisotope labelling techniques.

α-DFMA had little overall effect on the content of polyamines, except in increasing bound norspermidine, supporting the observations that ADC activity plays a relatively minor role in polyamine synthesis in this species (Voigt et al., 2000). The effective concentration of α-DFMA could be significantly lowered by endogenous arginase in the cells which could hydrolyse DFMA to DFMO. Since arginine decarboxylase is a multigene family, isoforms could be present in the cell extracts that are inhibitor insensitive, as reported by us previously in *Arabidopsis* (Bagni et al., 2006), and therefore account for the increase in cell division associated with an increase in bound norspermidine levels.

It is also noteworthy that there is no apparent parallelism between the cellular concentrations of putrescine derivatives and those of diaminopropane derivatives in algae (Hamana and Matsuzaki, 1982). The discrepancy is very clear in *E. gracilis* in the norspermine level which is high (23% of total polyamines) while spermine constitutes only 3%. The cellular level of spermine synthase may be very low but the activity of norspermine synthesis is high. These results support the view that, also in *C. reinhardtii*, putrescine derivatives and diaminopropane derivatives are synthesised by separate enzyme systems as previously evidenced for bacteria (Hobley et al., 2014).

In *Chlamydomonas mowusii* cultured on a carbon source, the major polyamine present was putrescine (85%) while spermidine (10%), spermine (5%), norspermidine (5%), norspermine (0.1%) and cadaverine (5%) were all detected (Harris, 2001). In the cell wall mutant of *Chlamydomonas* used in the current study again putrescine was the major polyamine (88%) with norspermidine as the second most abundant (11%). Spermine was only present in trace amounts while norspermine and cadaverine were not detected, however, unlike *C. mowusii*,

we detected trace levels of diaminopropane. The major point of difference between the analyses of *C. reinhardtii* cell wall mutant and that reported for *C. mowusii* was the level of total polyamines present, with the latter species having an almost three-fold higher level (6.8 μg g $wwt^{-1}$compared to 17.6 μg g $wwt^{-1}$). A possible explanation for this is the absence of cell walls in our strain which is known to be a significant source of polyamines in other plant tissues (Bassard et al., 2010).

Addition of exogenous polyamines reduced cell numbers in the order spermine>spermidine>norspermidine>putrescine, an effect which is related to the number of positive charges on the molecule and hence its uptake from the medium as supported by previous studies with the unicellular red alga *Porphyridium* which showed a similar trend (Scoccianti et al., 1989). In the presence of the spermidine synthase inhibitor, cyclohexylamine (CHA), both spermidine and norspermidine levels were reduced and the level of putrescine increased suggesting a possible effect of this inhibitor on the norspermidine biosynthetic enzymes (Fig. 4) despite the low sequence homology with bacterial spermidine synthase (Hobley et al., 2014). In *Chlorella*, spermidine is the major polyamine (52%) with significant levels of putrescine also present (45%). The level of norspermidine in this organism (50 nmol g $fw^{-1}$) (Hamana and Matsuzaki, 1982) is significantly lower than that reported here for *Chlamydomonas* (633 nmol g $fw^{-1}$). Only norspermidine significantly stimulated culture growth and was able to reverse the inhibitory effects of the CHA inhibitor.

In conclusion, the results reveal that the relative contribution of both ODC and ADC to polyamine formation in *Chlamydomonas* (CC-406 cell-wall mutant) is in contrast to the ADC route reported for *Chlorella vulgaris* to which it is taxonomically related (both *Chlorophyta*). If the present data will be confirmed also by analogous studies on the *Chlamydomonas* wild-type, this will imply that generic statements for taxonomic groups, regarding their biochemistry of polyamines cannot be made and that significant species differences exist that modulate their endogenous levels. The precise role of norspermidine in the genetic regulation of the algal cell cycle remains to be elucidated and is a potential key area for future work.

**Contribution**

AT and NA designed and conducted the experiments and biochemical measurements; GG designed the study, wrote and revised the manuscript. All of the authors read and approved the manuscript.

**Conflict of interest**

None

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**Figure Legends**

**Fig. 1** Effect of (**a**) DFMO and (**b**) DFMA on cell division. The results are expressed as a % increase relative to control samples where control cell numbers correspond to 20.9 ± 1.5 x $10^6$ cells $mL^{-1}$(48h), 25.6 ± 0.6 x $10^6$ cells $mL^{-1}$ (72h) and 30.6 ± 0.4 x $10^6$ cells $mL^{-1}$ (120h). Values are the mean ± SD (n=4).

**Fig. 2** Effect of adding 0.1 mM exogenous putrescine, spermidine, norspermidine and spermine on culture growth. Norspermidine and spermine values are statistically significantly different (Student's *t*-test, $p<0.05$) from control data starting from 48h of culture while putrescine data starting from 72h of culture. Values are the mean ± SD (n=4).

**Fig. 3** Effect of adding CHA inhibitor (0.1 mM) in the presence or absence of 0.1mM putrescine, spermidine, norspermidine or spermine on culture growth. Control and norspermidine mine values are statistically significantly different (Student's *t*-test, $p<0.05$) from CHA data starting from 48h of culture while putrescine and spermine data starting from 72h of culture. Values are the mean ± SD (n=4).

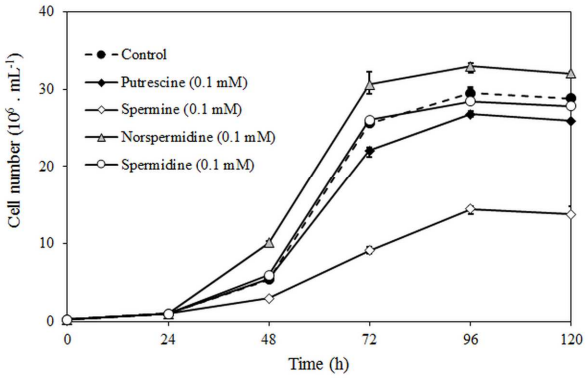
**Fig. 4** Proposed norspermidine biosynthetic pathway in *Chlamydomonas reinhardtii*. ADC, ariginine decarboxylase; CHA, cyclohexylamine; DABA:2KG AT, diaminobutyrate:2-ketoglutarate aminotransferase; DABA DC, diaminobutyrate decarboxylase; DFMA, α–difluoromethylarginine; DFMO α-difluoromethylornithine; ODC, ornithine decarboxylase; PAO, polyamine oxidase. The numbers 1 and 2 indicate the two possible utilization routes of ornithine.

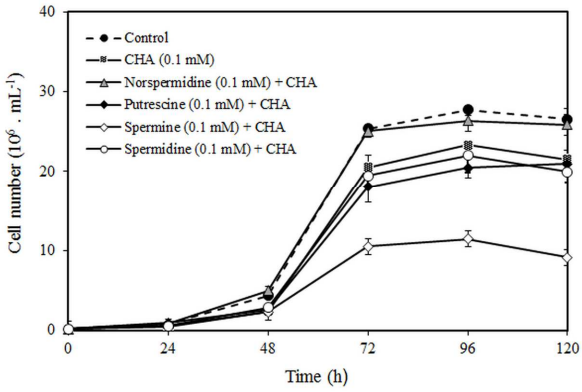
**Table 1.** ODC and ADC enzyme activities (pmol $2h^{-1}$mg $protein^{-1}$) in *Chlamydomonas reinhardtii.* Algae were cultured for 72h in media containing 5mM α–DFMO, α–DFMA or both inhibitors together and both supernatant and pellet fractions were assayed for activity. The star symbol indicates a statistically significant difference (Student's *t*-test, $p<0.05$) between treatment and control data. Values are the mean ± SD of four samples.

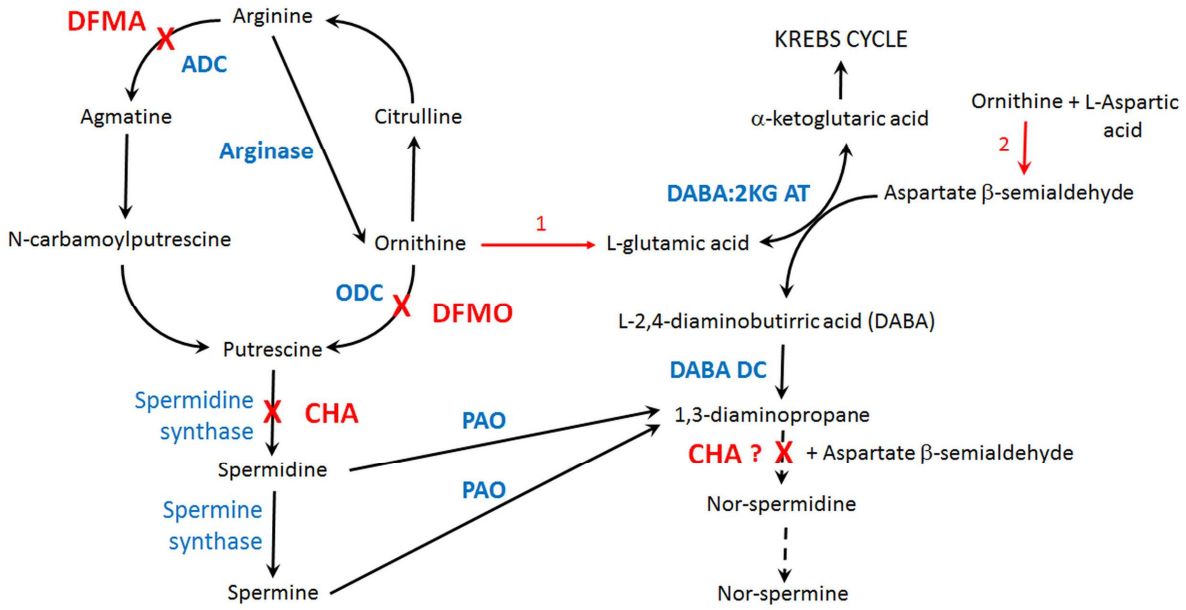
| | ODC activity | | | | ADC activity | | | |
|---|---|---|---|---|---|---|---|---|
| **Samples** | **Supernatant** | **Inhibition (%)** | **Pellet** | **Inhibition (%)** | **Supernatant** | **Inhibition (%)** | **Pellet** | **Inhibition (%)** |
| **Control** | 18.39±2.28 | - | 11.97±0.11 | - | 3.78±0.49 | - | 2.19±0.47 | - |
| **α–DFMO** | 15.76±0.97* | 14 | 6.45±0.28* | 46 | 4.42±0.15 | - | 1.34±0.07* | 39 |
| **α–DFMA** | 18.67±0.10 | - | 9.20±0.77* | 23 | 2.15±0.76* | 43 | 1.63±0.01* | 26 |
| **α–DFMO + α–DFMA** | 14.76±1.84* | 20 | 6.46±0.88* | 46 | 2.81±0.55* | 26 | 1.78±0.19* | 19 |

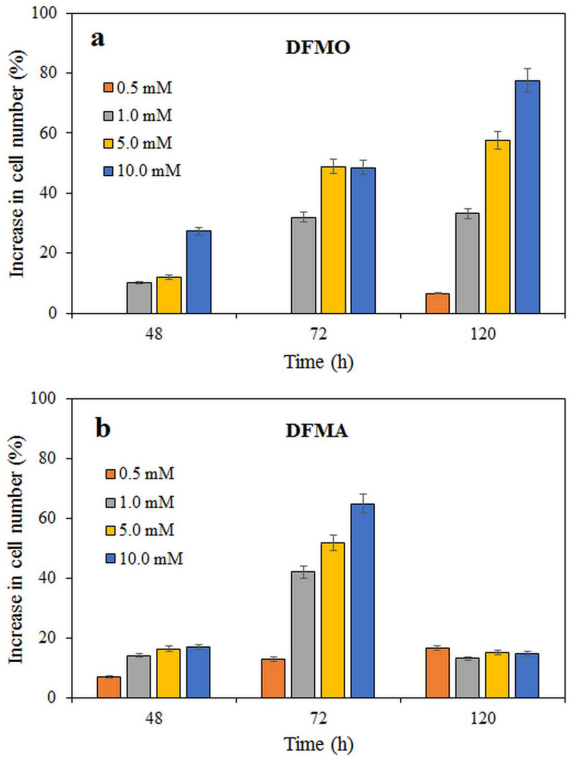
**Table 2.** Free and covalently bound (PCA-soluble fraction) polyamine content (nmol $gfw^{-1}$) in *Chlamydomonas reinhardtii* cells after subculture for 72h in media containing 5 mM α-DFMO, α-DFMA or both inhibitors together. Covalently bound polyamines in the PCA-insoluble fraction were detected in trace levels (<0.1 %, data not shown). The values in parentheses are the % values of each polyamine within the free or bound state. The star symbol indicates a statistically significant difference (Student's *t*-test, $p<0.05$) between treatment and related (free or bound) control data. Values are the mean ± SD of four samples.

| | **Control** | | **α-DFMO** | | **α-DFMA** | | **α-DFMO + α-DFMA** | |
|---|---|---|---|---|---|---|---|---|
| **Polyamines** | **Free** | **Bound** | **Free** | **Bound** | **Free** | **Bound** | **Free** | **Bound** |
| **Diaminopropane** | 1.4±0.4 (<0.1%) | 17.5±4.0 (1.0%) | 1.1±0.2 (<0.1%) | 12.4±1.7 (0.6%) | 1.7±0.3 (<0.1%) | 23.4±3.4 (1.5%) | 1.3±0.4 (<0.1%) | 13.1±0.1 (0.6%) |
| **Putrescine** | 4470.3±314.7 (88%) | 1594.7±25.5 (93.3%) | 2577.6±215.0* (74.6%) | 1520.5±22.4 (70.0%) | 4394.9±449.9 (86.9%) | 1418.4±82.1 (88.9%) | 2430.4±184.6* (72.1%) | 1380.9±114.9* (68.9%) |
| **Norspermidine** | 540.0±38.3 (10.6%) | 93.8±10.3 (5.5%) | 876.1±37.0* (25.4%) | 638.5±71.2* (29.4%) | 587.9±61.8 (11.6%) | 153.2±2.6* (9.6%) | 939.1±64.7* (27.9%) | 609.1±23.6* (30.4%) |
| **Spermidine** | 65.9±15.7 (1.3%) | 1.7±0.3 (0.1%) | Not detected | Not detected | 72.7±20.7 (1.4%) | Not detected | Not detected | Not detected |
| **Spermine** | 1.2±0.1 (<0.1%) | 1.1±0.1 (<0.1%) | 1.0±0.1 (<0.1%) | 0.2±0.0* (<0.1%) | 0.9±0.1 (<0.1%) | 0.8±0.1 (<0.1%) | 1.0±0.3 (<0.1%) | 1.0±0.2 (<0.1%) |
| **Total** | 5078.8 (74.8%) | 1708.8 (25.2%) | 3455.8 (61.4%) | 2171.6 (38.6%) | 5058.1 (76.0%) | 1595.8 (24.0%) | 3371.8 (62.7%) | 2004.1 (37.3%) |
| **Total Free +Bound** | **6787.6** | | **5627.4** | | **6653.9** | | **5375.9** | |









**Highlights**

Manuscript Title: "**Effect of ornithine decarboxylase and norspermidine in modulating cell division in the green alga *Chlamydomonas reinhardtii***".

by Annalisa Tassoni, Nahid Awad and Gareth Griffiths

- Putrescine and norspermidine were the most abundant polyamines in free and membrane-bound fractions
- ODC activity was almost five times higher than ADC activity being the major route of putrescine synthesis.
- α-DFMO inhibited membrane associated ODC activity simultaneously stimulating cell division
- Addition of norspermidine stimulated cell division mimicking the effects observed in the presence of DFMO
- Norspermidine reversed the inhibitory effects of cyclohexylamine on growth

**Manuscript "Effect of ornithine decarboxylase and norspermidine in modulating cell division in the green alga *Chlamydomonas reinhardtii*" by Tassoni et al.**

**Contribution**

AT and NA designed and conducted the experiments and biochemical measurements; GG designed the study, wrote and revised the manuscript. All of the authors read and approved the manuscript.