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Protistology

Cha 1, a DNA incertional transformant of the green alga *Chlamydomonas reinhardtii* with altered chemotaxis to ammonium

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Summary

DNA insertional mutagenesis and screening of the unicellular green alga *Chlamydomonas reinhardtii* was employed to isolate *cha1,* a stable transformant having an altered chemotactic behaviour to ammonium. Mutant *cha1* did not exhibit chemotaxis to ammonium when vegetative cells were grown in the media supplemented with nitrogen-containing compounds that were transported into the *Chlamydomonas* cells by a specific transport system (arginine, urea). In contrast, the cells demonstrated chemotactic responses to ammonium in the ammonium-containing media. These results were interpreted so that the presence of particular transport systems, according to their affinity and specificity for ammonium, is responsible for chemotactic signalling in *Chlamydomonas*.

Key words: *Chlamydomonas reinhardtii,* DNA insertional mutagenesis, chemotaxis

Introduction

Inorganic ions accumulated in cells and unicellular organisms perform nutritional, signaling and storage functions. Ammonium is often a preferred source of nitrogen for many organisms, including the soil phototrophic protist *Chlamydomonas reinhardtii*. However, nitrogen is commonly considered as a limiting nutrient, and organisms have developed a variety of adaptations that enable them to respond to their internal nutritional status as well as to the external availability of ammonium. Besides, many motile organisms have evolved an additional adaptation such as chemotaxis that allows them to move towards ammonium.

Chemotactic behaviour is the oriented movement of a cell in response to a chemical signal. Many eukaryotic microbes are motile and show chemotactic behaviour (Devreotes and Zigmond, 1988; Arkowitz, 1999; Frenchel and Blackburn, 1999). Very little is known about these signal transduction pathways in

flagellated cells. We have used the unicellular green biflagellate alga *Chlamydomonas* to investigate flagellate chemotaxis. For this protist, the tools for genetic and molecular studies are well developed. In addition, databases with sequence information from more than 130,000 EST clones are available (Shrager et al., 2003).

Chlamydomonas vegetative cells are attracted to the preferred nitrogen source, ammonium (Sjoblad and Frederikse, 1981). This alga has different ammonium carriers that vary in their kinetic properties and regulatory mechanisms (Franco et al., 1988). Recent studies have revealed that the chemotactic response of *C. reinhardtii* towards ammonium is altered during gametogenesis (Ermilova et al., 2003a, 2003b). Unlike vegetative cells and mating incompetent pregametes (generated by incubation of vegetative cells without a nitrogen source in the dark), mature gametes do not show chemotaxis towards ammonium. But little is known about the mechanisms by which attraction of motile cells to ammonium is evoked.

In this paper we describe the isolation and preliminary characterization of a mutant *cha1* from C. *reinhardtii,* which has an altered chemotaxis to ammonium. Our data suggest an overlap between the pathways that control ammonium transport and chemotaxis to ammonium in this protist.

Material and methods

Strains and culture conditions

Chlamydomonas reinhardtii wild-type strain CC-124 (mt^-), obtained from the *Chlamydomonas* Culture Collection at Duke University, USA, was used as the tester strain. Another wild-type strain tested was CC-620 (mt^+), obtained from S. Purton, University College London, GB. Cells were grown at 22° C under a 12-h light/12-h dark regime in Tris acetate phosphate (TAP) medium (Gorman and Levin, 1965) or in an acetate-free TAP (TMP) medium. Cellular growth was estimated as number of cells per 1 ml.

Chemotaxis assay

Chemotactic responses were tested by counting the number of cells that in darkness swam into rectangular capillaries (260 μm × 450 μm) filled with 3 μl of medium containing NH_4NO_3 (5 mM). This number was compared to the number of cells entering capillaries filled with ammonium-free medium (Ermilova et al., 1998). Capillaries were closed at one end with Parafilm. The other end was submerged in the cell suspension for 10 min at 22°C.

The chemotaxis index (CI) was calculated using the following equation:

$$CI = \frac{\text{number of cells entering capillaries filled with medium containing } NH_4NO_3}{\text{number of cells entering capillaries filled with } NH_4\text{-free medium}}$$

Data are the means of triplicate determination from representative experiments.

Transformation

The pSP124S plasmid kindly provided by S. Purton (University College London, GB) was used for transformation. Before transformation, the plasmid DNA was linearized with *Kpn*I. To prepare the cells for transformation, the cell wall was removed by treatment with autolysin that was produced as described (Harris, 1989). The DNA was introduced into the cells by using the glass bead method (Kindle, 1990). For direct selection of zeomycin resistant transformants, cells were agitated with glass beads and DNA, diluted in 20 ml TAP liquid medium and left to express the *ble* gene by incubating at 25° C in the light for 15-18 h with gentle shaking. Cells were then pelleted by centrifugation, resuspended in 5 ml of TAP containing 0.5 % molten agar, and poured onto the surface of an agar plate with 10 μg/ml zeomycin (Cayla-France).

Genetic analysis

Tetrad analysis was performed using standard techniques (Levine and Ebersold, 1960). Gametes of *cha1* (*cha1*mt^-) and CC620 (*cha1*$^+mt^+$) were induced in nitrogen-free medium upon continuous illumination, mated and the mating mixture was spread onto zygospore maturation plates (3% agar TAP). After maturation, the zygospores were isolated and transferred onto germination plates (1,5 % agar TAP). Colonies that appeared on these agar TAP plates were spread again onto new TAP agar plates to obtain single colonies. Progeny colonies were scored for resistance to zeomycin, motility, chemotactic phenotype and mating type.

Results and discussion

Isolation of mutant defective in chemotaxis to ammonium

Chlamydomonas exhibits chemotaxis to ammonium and some sugars (Ermilova et al., 1998). In one set of mutants previously identified chemotaxis to different sugars was blocked (Ermilova et al., 2000). For the isolation of a new set of mutants defective in chemotaxis to ammonium, we employed insertional mutagenesis. The mutants were generated using an insertional mutagenesis protocol in which the strain CC124 was

transformed with the pSP124S plasmid carrying *ble* gene (Lumbreras et al., 1998). The *ble* gene originates from the tallysomycin-producing actinomycetes species *Streptoallotiechus hindustanus* and encodes a 13,7 kDa protein conferring resistance to tallysomycin and related antibiotics including zeomycin. The *ble* coding region was fused to the 5' and 3' regulatory regions of the *Chlamydomonas RBS2* gene to create the plasmid that allows direct selection for zeomycin-resistant transformants (Stevens et al., 1996). At the first step of screening for chemotaxis mutants, transformants were selected as colonies that were able to grow on nutrient agar containing zeomycin (Fig. 1). In five independent experiments 2240 colonies were obtained. At the second step, the mutants with lower growth than the recipient strain CC124 were selected on TAP medium. All strains were fully motile. These strains were then indi-vidually examined for chemotactic defects. From 41 strains examined, one was not attracted to ammonium if vegetative cells were grown in TMP-N medium suplemented with arginine. This transformant was named *cha1* (mutant in chemotaxis to ammonium).

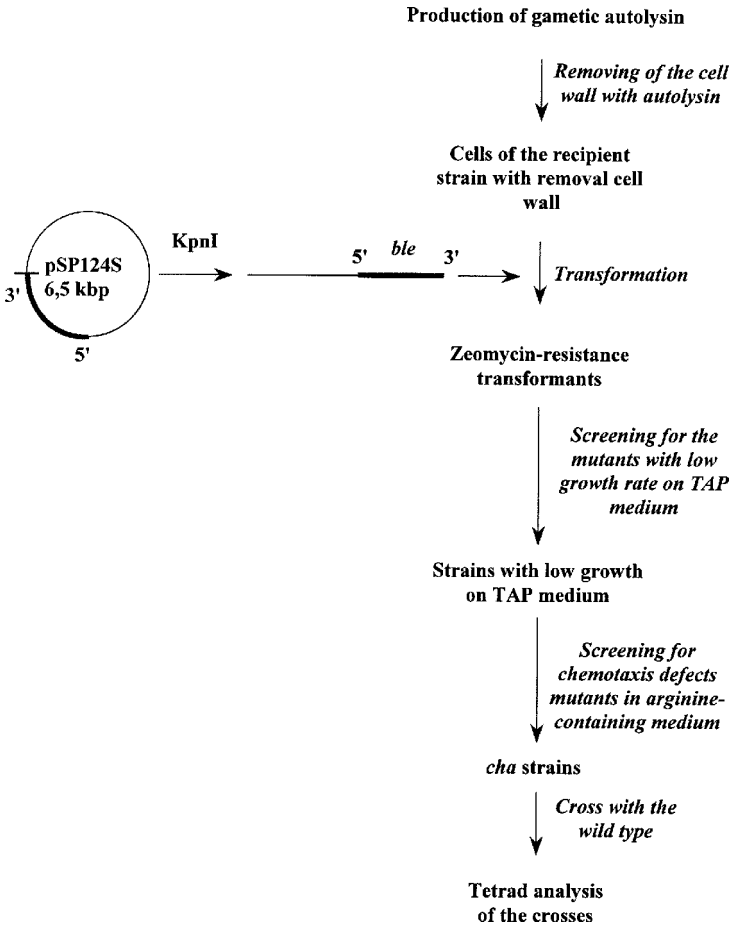

Fig. 1. Scheme of generation and selection of *C. reinhardtii* mutants with altered chemotaxis to ammonium.

Phenotypic characterization of *cha1* mutant

Mature gametes did not show chemotaxis to ammonium (Ermilova et al., 2003a). Amino acids did not prevent the formation of chemotactically inactive gametes when vegetative cells did not utilize them (Ermilova et al., 2003b). However, arginine was assimilated by the mutant *cha1* and supported the growth of vegetative cells (Fig. 2). Therefore, loss of chemotaxis to ammonium in arginine-containing medium is not a result of gamete formation induced by nitrogen starvation.

Arginine is known to be the only amino acid, which can be transported into the cells by a specific transport system (Kirk and Kirk, 1978). If the strain *cha1* were transferred on TAP medium, cells started to respond to ammonium chemotactically (Fig. 3). Chemotaxis index for the mutant was lower than for wild type. In addition, the growth in ammonium media of *cha1* was lower than that of the wild type cells. These data suggest that *cha1* might be altered in the transport of ammonium. Ammonium transport is a highly regulated carrier-mediated process in *C. reinhardtii*. One system is constitutive and responsible for ammonium transport at high concentrations, whereas the others are ammonium repressible and responsible for providing ammonium to alga when ammonium is present at low concentrations in the medium (Franco et al., 1988).

In a parallel experiment, we tested the effects of urea on chemotaxis of *cha1* (Table 1). Vegetative cells grown in urea-containing medium completely lacked chemotaxis to ammonium. Urea is incorporated into *Chlamydomonas* cells by an active transport mechanism (Williams and Hodson, 1977). We propose that constitutive component for ammonium transport is likely to be blocked in the strain *cha1*. If nitrogen-containing compounds (arginine, urea) were transported into the mutant by a specific transport system, cells did not induce ammonium transporters that are ammonium-repressible in wild type. However, in the strain *cha1* this carrier(s) is active in the media with ammonium, probably because the mutant is in a situation of nitrogen starvation due to limiting ammonium transport. These data were supported by the experiment with alanine and glutamine (Table 1).

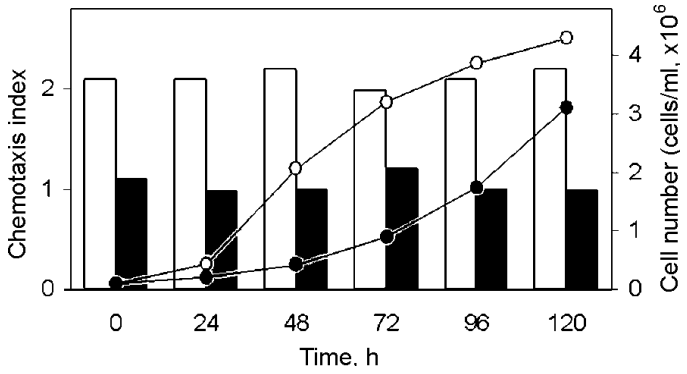

Fig. 2. Effects of L-arginine on cell growth and chemotaxis activity to ammonium of the CC124 and *cha1* strains. At the times indicated, cell number of the CC124 (○) and *cha1* (●), and chemotaxis index of the CC124 (□) and *cha1* (■) were determined.

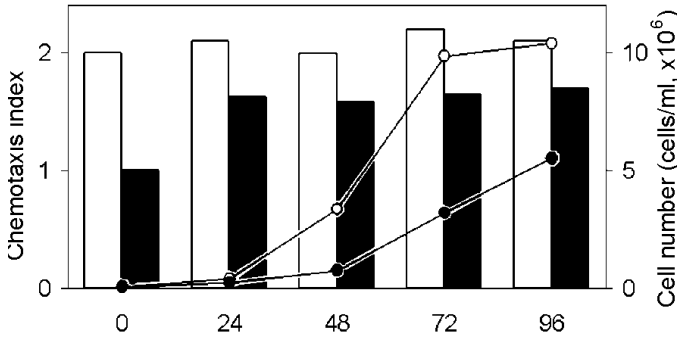

Fig. 3. Cell growth and chemotaxis to ammonium of the CC124 and *cha1* strains in TAP medium. At the times indicated, cell number of the CC124 (○) and *cha1* (●), and chemotaxis index of the CC124 (□) and *cha1* (■) were determined. Vegetative cells were grown in TAP–N medium supplemented with L-arginine and transferred in TAP medium at time 0.

Table 1. Effects of nitrogen-containing compounds on cell growth and chemotaxis to ammonium of the *cha1* mutant.

Medium	Chemotaxis to ammonium	Cell growth
TAP	+	+
Alanine	+	+
Glutamine	+	+
Urea	–	+

Notes: vegetative cells grown in TAP–N medium supplemented with L-arginine were transferred in TAP medium or TAP–N medium supplemented with various nitrogen-containing compounds. After 24, 48 and 72 h of incubation, cell number and chemotaxis index to ammonium were determined.

Cells started to respond to ammonium on the media supplemented with amino acids. As reported previously (Piedras et al., 1992), C. *reinhardtii* cells are able to deaminate the L-amino acids extracellularly by a non-specific L-amino acid oxidase. As a result, ammonium is generated and transported by inducible carrier(s).

Genetic characterization of *cha1* mutant

To test how many components were impaired in the mutant, the strain *cha1* (*cha1*, mt^-) was investigated by genetic analysis. The transformant was crossed with a wild type strain (*cha1*$^+$, mt^+). The mutant phenotype segregated as a single nuclear mutation, showing a 2:2 inheritance pattern in each tetrad. In each case, the mutant allele segregated independently of the mating-type locus on linkage group VI (Harris, 1989). The genetic analysis demonstrates that the alteration of chemotactic responses to ammonium is due to a mutation at a single locus rather than at multiple loci.

Ammonium transporters in C. *reinhardtii* belong to a multigenic family, Mep/Amt, membrane proteins highly conserved in Bacteria, Archae, fungi, plants and invertebrates (Marini et al., 1997). *Saccharomyces cerevisiae* synthesizes three Mep proteins but only the high affinity ammonium permease, Mep2, acts as an ammonium sensor, generating a signal to regulate differentiation in response to ammonium limitation (Lorenz and Heitman, 1998; Lengler et al., 2000). This example illustrates that ammonium transporter appears to be constituent of signalling pathway. We suggest that ammonium transporter(s) from *Chlamydomonas* acts in a similar manner to sense ammonium ions and induce, via an as-yet-unidentified signal transduction cascade, behavioural response to ammonium. Impor-tantly, further molecular characterization of *cha1* mutant defective in chemotactic responses to ammonium will provide a basis for eluci-dation of the ammonium transporter's function in the regulation of chemotactic signal flux.

Acknowledgements

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