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Sac1, a putative regulator that is critical for survival of *Chlamydomonas reinhardtii* during sulfur deprivation

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The *sac1* mutant of *Chlamydomonas reinhardtii* is aberrant in most of the normal responses to sulfur limitation; it cannot synthesize arylsulfatase, does not take up sulfate as rapidly as wild-type cells, and does not synthesize periplasmic proteins that normally accumulate during sulfur-limited growth. Here, we show that the *sac1* mutant dies much more rapidly than wild-type cells during sulfur deprivation; this emphasizes the vital role of the acclimation process. The loss of viability of the *sac1* mutant during sulfur deprivation is only observed in the light and is mostly inhibited by DCMU. During sulfur-stress, wild-type cells, but not the *sac1* mutant, downregulate photosynthesis. Thus, death of the *sac1* mutant during sulfur deprivation is probably a consequence of its inability to downregulate photosynthesis. Furthermore, since *SAC1* is necessary for the downregulation of photosynthesis, the process must be highly controlled and not simply the result of a general decrease in protein synthesis due to sulfur limitation. Genomic and cDNA copies of the *SAC1* gene have been cloned. The deduced amino acid sequence of Sac1 is similar to an *Escherichia coli* gene that may involved in the response of *E.coli* to nutrient deprivation.

Keywords: nutrient stress/photosynthesis

Introduction

The unicellular green alga *Chlamydomonas reinhardtii* can monitor and adjust to changes in the nutrient status of its environment. To elucidate the ways in which this alga senses and acclimates to sulfur limitation, we have defined some of the physiological and biochemical changes that accompany sulfur limitation (de Hostos *et al.*, 1988, 1989; Yildiz *et al.*, 1994) and have isolated and characterized mutants that are aberrant in these responses (Davies *et al.*, 1994).

Organisms respond to the loss of a commonly available form of a nutrient by accessing alternative sources of the limiting nutrient and importing the nutrient more efficiently. Alternative nutrient sources may be located within the cell or in the environment. Macromolecular protein complexes within the cell may be sacrificed and the nitrogen and sulfur present in the amino acids mobilized during nutrient limitation. For example, when *Synechococcus* sp. strain PCC7942 is starved for either sulfur or nitrogen, the light-harvesting phycobilisomes are degraded (Yamanaka and Glazer, 1980; Collier and Grossman, 1992), while in *Lemna* the ribulose bisphosphate (RuBP) carboxylase is degraded (Ferreira and Teixeira, 1992). These proteins are among the most abundant within these cells, contain a significant amount of sulfur and nitrogen and are not required during nutrient deprivation when anabolic processes are markedly reduced. It has been estimated that the sulfur released in the form of amino acids during degradation of RuBP carboxylase in *Lemna* would establish an intracellular sulfur concentration of 10 mM (Ferreira and Teixeira, 1992).

Efficient assimilation of alternate forms of a nutrient from outside the cell may also be critical for surviving periods of nutrient limitation. For example, most micro-organisms prefer to use ammonia; however, when ammonia is not available the organisms develop the capacity to assimilate nitrate (Caboche and Rouzé, 1990; Crawford and Arst, 1993). This newly developed capacity is due to increased transcription of the genes encoding proteins required for nitrate assimilation (nitrate reductase, nitrite reductase and the nitrate transport system). Furthermore, extracellular phosphatases and sulfatases may be synthesized when the cells are limited for phosphorus and sulfur, respectively (Scott and Metzenberg, 1970; Apte *et al.*, 1974; Adachi *et al.*, 1975; Lien and Schreiner, 1975; Goldstein *et al.*, 1988). These enzymes increase nutrient availability by hydrolyzing phosphate or sulfate from extracellular organic molecules that are not readily assimilated.

In addition to exploiting alternative sources of a nutrient, micro-organisms often increase their ability to import the preferred form of the nutrient. Sulfate is the sulfur source preferred by most micro-organisms. When it becomes limiting, cyanobacteria, algae, fungi and plants increase synthesis of high affinity sulfate transport systems (Dreyfuss, 1964; Arst, 1968; Marzluf, 1970a; Breton and Surdin-Kerjan, 1977; Jensén and König, 1982; Ames, 1986; Green and Grossman, 1988).

During periods of extended, severe nutrient limitation, microbes stop cell division and dramatically alter their metabolism. The cessation of cell division is a requisite for survival during nitrogen or sulfur starvation of the yeast *Saccharomyces cerevisiae*. Yeast cells with a *bcy1* or *pde2* mutation are unable to halt cell cycle progression and die during nutrient deprivation (Toda *et al.*, 1987; Wilson and Tatchell, 1988). Yeast cells also alter their sugar metabolism during nutrient deprivation: instead of oxidizing sugars to provide energy for cell growth, the sugars are polymerized and stored as complex carbohydrates (Lillie and Pringle, 1980).

A number of features of the acclimation of *Chlamy-*

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Table I. Strains

Strain	Genotype
CC125	*mt⁺ nit1 nit2*
CC425	*mt⁺ cw15 nit1 nit2 arg7-8*
CC2267	*mt⁻ nit1*
ars5-1	*mt⁺ cw15 nit1 nit2 sac1::ARG7*
ars5-4	*mt⁺ cw15 nit1 sac1::ARG7*

Table II. Clones

Clone	Description
λ#3, λ#7	Separate EMBL3 clones of genomic DNA containing the *SAC1* gene
pJD160	6.5 kbp subclone containing the *SAC1* gene in pBluescriptKS+
pJD164	1.5 kbp subclone of pJD160 containing the *SAC1* gene in pBluescriptKS+
pJD165	4.5 kbp subclone of pJD160 containing the *SAC1* gene in pBluescriptKS+
pJD168	6.0 kbp subclone of pJD160 containing the *SAC1* gene in pBluescriptKS+
pJD236	3.5 kbp cDNA clone of *SAC1* in pGEM7
pMN24	*NIT1* genomic clone in pUC119 (obtained from P.Lefebvre)

domonas to nutrient limitation are similar to those observed for fungi and micro-organisms. For example, sulfur-limited *Chlamydomonas* cells stop dividing, accumulate starch (Ball *et al.*, 1990), induce arylsulfatase (Ars, an enzyme that cleaves sulfate from aromatic sulfates) (Lien and Schreiner, 1975; de Hostos *et al.*, 1988) and increase sulfate uptake (Yildiz *et al.*, 1994). However, there are also a number of differences; in particular, the signal transduction pathway that controls the responses of *Chlamydomonas* to nutrient limitation appears to be significantly different from that of fungi or bacteria (Davies *et al.*, 1994). Furthermore, unlike organisms that do not perform photosynthesis, *Chlamydomonas* must modulate photosynthetic activity and control the utilization and dissipation of excess light energy during nutrient limitation. Here we demonstrate that the *sac1* mutant, which is unable to acclimate to sulfur limitation, dies much more quickly than wild-type cells under conditions of sulfur deprivation. This decrease in viability is light-dependent and appears to reflect the inability of the mutant to decrease photosynthetic electron transport during sulfur limitation. The *SAC1* gene, which is critical for many of the cellular modifications that accompany sulfur deprivation, was isolated and analyzed. Its role in the acclimation of *Chlamydomonas* to sulfur limitation is discussed.

Results

Death of the sac1 mutant in sulfur-deficient medium

The strains and the plasmids that have been used in the work described here are listed in Tables I and II. The *sac1* mutant (strain *ars5-1*) was isolated from a mutagenized population of strain CC425 by screening for colonies that exhibited little or no Ars activity when the cells were grown under sulfur-limiting conditions (Davies *et al.*, 1994). Since *ars5-1* is defective in many aspects of the sulfur-stress acclimation response (Davies *et al.*, 1994),

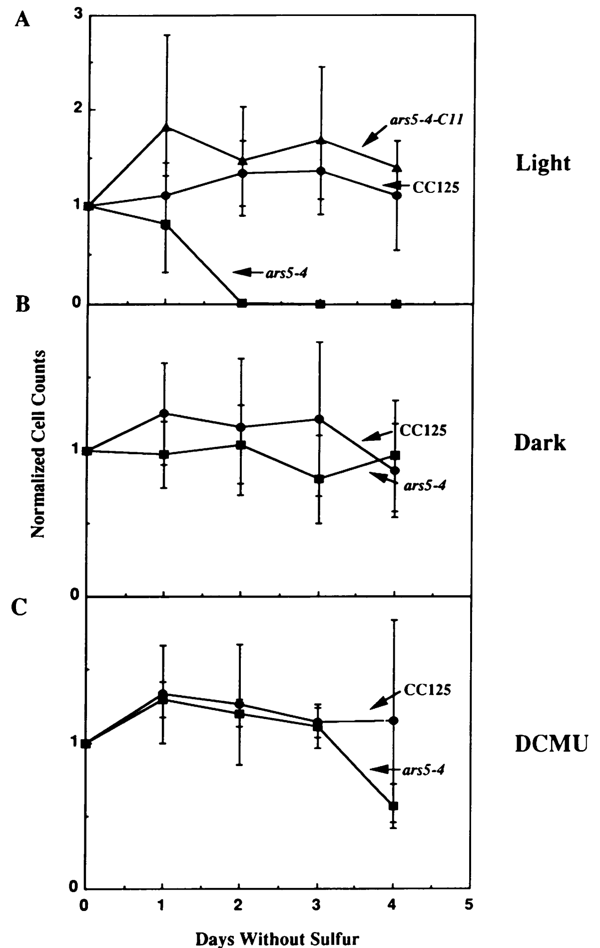


Fig. 1. Photosynthesis kills sulfur-starved *sac1* cells. CC125 (wild-type) (●), *ars5-4* (*sac1*) (■) and *ars5-4-C11* (complemented *sac1*) (▲) cells were grown to mid-logarithmic phase, washed twice with –S medium and incubated in –S medium (**A**) in the light, (**B**) in the dark and (**C**) in the light with 3 μM DCMU. Cell viability was determined by vital staining of the cells (see Materials and methods). The cell number was normalized to the value on day 0. The error bars represent the standard deviations from the mean.

Sac1 may function as a sensor of the sulfur status of the environment or as a component of the signal transduction chain just downstream of the sensory apparatus.

Physiological changes that occur during sulfur deprivation are thought to enable cells to survive periods when sulfur becomes unavailable. To test this hypothesis, we compared the viability of wild-type cells (CC125) and *sac1* cells (*ars5-4*, the product of a cross CC2267×*ars5-1*) maintained in the light in medium lacking sulfur (Figure 1A). Actively growing cells were washed and resuspended in medium devoid of sulfur, and cell viability was monitored by vital staining with methylene blue and phenosafarinin. The wild-type strain lost little pigmentation and exhibited little or no cell death. In contrast, *sac1* cells became chlorotic and died within 3 days of resuspension in medium lacking sulfur. These results demonstrate that a functional *SAC1* gene is critical for surviving sulfur deprivation in the light.

Nutrient limitation can lead to a reduction in photosynthesis via the rapid and specific inactivation of photosynthetic electron transport (Collier *et al.*, 1994). A decline in photosynthesis may be part of a generalized mechanism

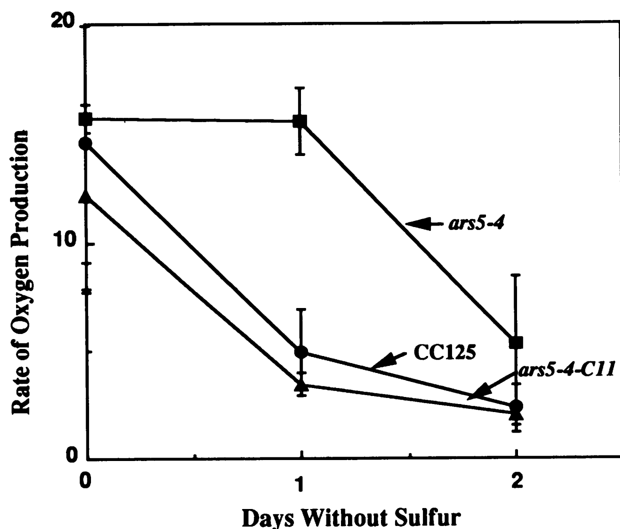


Fig. 2. The *sac1* mutant does not downregulate photosynthesis when starved for sulfur. Photosynthesis in –S incubated CC125 (wild-type) (●), *ars5-4* (*sac1*) (■) and *ars5-4-C11* (complemented *sac1*) (▲) cells was monitored by the measurement of oxygen evolution (nmol O_2 produced min^{-1} 10^6 $cells^{-1}$). The error bars represent the standard deviations from the mean.

that protects photosynthetic organisms from the damaging effects of toxic oxygen species that would tend to form in the light during periods of reduced anabolic activity. As shown in Figure 2, a marked decline in photosynthetic activity during the first 24 h of sulfur deprivation is apparent in wild-type *Chlamydomonas* cells. Since the *sac1* mutant is unable to survive sulfur deprivation in the light and does not exhibit several of the responses observed in wild-type cells that are placed in sulfur-deficient medium (Davies *et al.*, 1994), we speculated that this strain could not downregulate photosynthesis in response to sulfur deprivation and that continued photosynthesis under these conditions was toxic. Indeed, after 24 h in –S medium there is essentially no decrease in photosynthetic oxygen evolution in the *sac1* mutant. Hence, the decrease in photosynthesis within the first 24 h of sulfur stress appears to be a regulated process that requires the *SAC1* gene product, and it is not simply the result of stalled protein synthesis due to nutrient limitation. However, after 48 h of sulfur starvation (Figure 2), oxygen evolution in the *sac1* strain has decreased to the same extent as in wild-type cells. This decrease is most likely the result of inhibited protein synthesis caused by sulfur limitation.

To test whether the *sac1* mutant's inability to regulate photosynthetic electron transport was responsible for its death, we monitored cell viability during sulfur deprivation in the dark and in the presence of DCMU, a herbicide that blocks electron transport at photosystem II. Dark-maintained *sac1* cells survived sulfur starvation as well as wild-type cells incubated in either the light or the dark (Figure 1B). The addition of DCMU to 3 μM also rescued the mutant cells (Figure 1C). These results indicate that the continued operation of photosynthetic electron transport in the *sac1* mutant during sulfur limitation is lethal. Hence, a controlled reduction in photosynthetic electron transport appears to be critical for prolonging viability of *Chlamydomonas* during nutrient limitation in the light.

Cloning of the SAC1 gene

The *sac1* mutation in *ars5-1* was generated by transformation of CC425 (*cw15 arg7-8*) with a construct containing the *ARG7* gene flanked on both sides by portions of the *ARS2* gene (5′-*ARS2/ARG7/ARS2*-3′) (Davies *et al.*, 1994). Integration of the introduced DNA into the nuclear chromosome of *Chlamydomonas* occurs primarily by non-homologous recombination (Kindle *et al.*, 1989) and introduces mutations randomly throughout the genome. To determine whether integration of 5′-*ARS2/ARG7/ARS2*-3′ caused the *sac1* lesion in *ars5-1*, we tested for co-segregation of the mutant phenotype and the introduced DNA. The mutant was crossed with CC2677 (*nit1-305*) that has a wild-type *SAC1* gene; in all 21 of the random progeny examined the introduced 5′-*ARS2/ARG7/ARS2*-3′ DNA and the mutant phenotype co-segregated (data not shown).

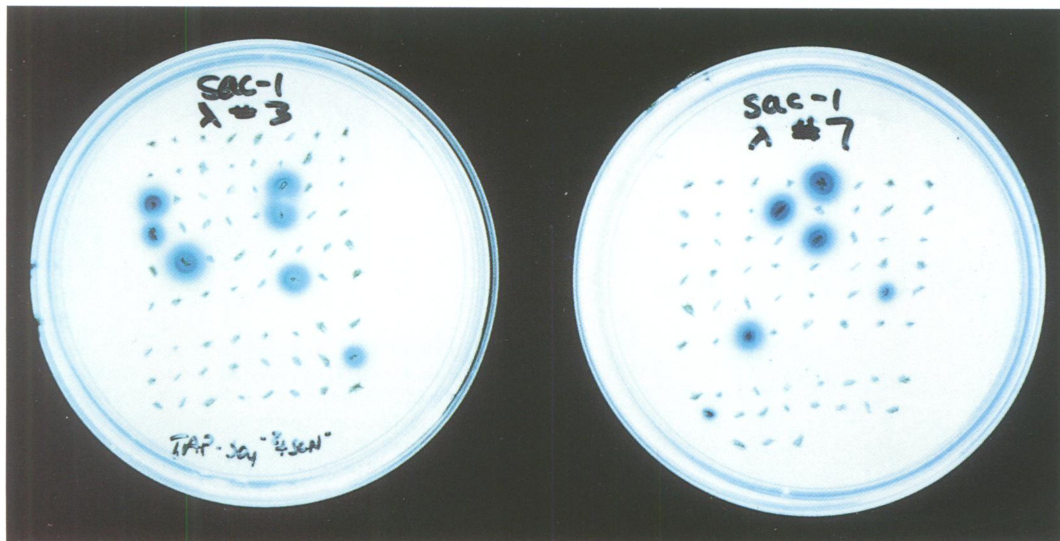
To isolate the mutated copy of the *SAC1* gene, a recombinant library in λEMBL3 was prepared from *ars5-1* genomic DNA and screened for phage that hybridized to both *ARG7* and *ARS2*. From one positive clone, a DNA fragment adjacent to the inserted DNA was isolated and used to screen a recombinant library constructed from wild-type genomic DNA. The positive clones isolated from the wild-type library were tested for complementation of the *sac1 nit1* mutant strain (*ars5-4*) by co-transformation with the *NIT1* gene. Complementation of the mutant phenotype was examined by assaying the transformants for Ars activity during growth on sulfur-deficient medium (Figure 3A). Two of the λ clones, designated λ#3 and λ#7, restored the ability of *ars5-4* to express Ars during growth in sulfur-deficient medium. Complementation frequencies were similar with both clones (13/177 for λ#3 and 12/124 for λ#7). In the control experiment in which only the *NIT1* gene was introduced into *ars5-4*, none of the transformants expressed Ars activity. Restriction sites on the two complementing λ clones revealed an overlapping region of ~6.5 kbp. A 6.5 kbp *Sal*I fragment containing this overlapping sequence was subcloned from λ#7 to generate pJD160. Introduction of pJD160 into *ars5-4* resulted in complementation of the mutant phenotype. Subclones of pJD160 (pJD164, pJD165 and pJD168, Figure 3B) were unable to complement the mutant phenotype.

Transformants of *ars5-4* expressing Ars activity were tested for the presence of an extra copy of the putative *SAC1* gene by DNA gel blot analysis. Figure 4 shows that the DNA of CC125, the wild-type strain, contains a single 8 kbp *Sal*I fragment that hybridizes with the *SAC1* sequence. CC425, the untransformed parental strain, also contains this 8 kbp *Sal*I fragment (data not shown). In contrast, the *SAC1* sequence hybridizes to a 5.0 kbp *Sal*I fragment in *ars5-4*, indicating that a portion of the *SAC1* gene must have been lost during the integration of the 5′-*ARS2/ARG7/ARS2*-3′ DNA. The deletion of genomic DNA during the integration of exogenous DNA into *Chlamydomonas* chromosomes has been previously observed (Tam and Lefebvre, 1993). In the complemented strains, two *Sal*I fragments hybridized to the *SAC1* sequence. These strains contain the 5.0 kbp *Sal*I fragment present in *ars5-4* plus an additional *SAC1* sequence that was introduced during the transformation. In *ars5-4-C1*, the strain that was complemented with λ#7, there is an

A

λ#3

λ#7



B

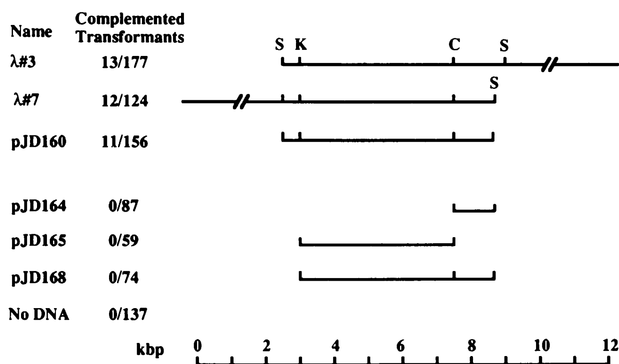


Fig. 3. Complementation of the *sac1* mutant. (**A**) Colonies of *ars5-4* (*sac1 nit1*) transformed with pMN24 (containing the *NIT1* gene) and λ#3 or λ#7 (containing the *SAC1* gene) were grown on –S medium for 4 days and sprayed with 5-bromo-4-chloro-3-indolyl sulfate (XSO_4) (Davies *et al.*, 1994). The *NIT1* gene serves as the selectable marker for transformation, allowing growth on medium containing nitrate as the sole source of nitrogen. Colonies with an intact *SAC1* gene show induction of Ars expression (identified by the blue color surrounding the colony) on –S medium. (**B**) Schematic maps of clones used in complementation experiments. Restriction sites are designated as S (*Sal*I), K (*Kpn*I) and C (*Cla*I). All the restriction sites are known to be in the genomic sequences with the exception of the left-hand *Sal*I site on λ#3 and the right-hand *Sal*I site on λ#7, which are very close to or in the multiple cloning region of the EMBL3 vector.

additional hybridizing *Sal*I fragment of 6.5 kbp, while in *ars5-4-C11* and *ars5-4-C12*, strains complemented with λ#3, the additional hybridizing band is 7.0 kbp. These are the expected sizes for the introduced copies of *SAC1* based on restriction map analysis of the insert DNA in λ#3 and λ#7 (see Figure 3B). However, the largest *SAC1* hybridizing *SalI* fragment in the lambda clones is 7 kbp; ~1 kbp smaller than the genomic fragment in CC125. This size difference appears to be due to a polymorphism between CC125 and the strain used to construct the genomic library.

Expression and sequence analysis of the SAC1 gene

Three *SAC1* cDNA clones containing identical 3.5 kbp inserts were isolated using the insert from pJD160 as a probe from a recombinant library. The cDNA library was prepared from pooled RNA preparations from *Chlamydomonas* cells starved for sulfur for 1, 2 and 4 h. The insert was hybridized to gel blots of poly(A) RNA that was purified from a wild-type (CC125) and a *sac1* mutant (*ars5-4*) that were maintained in sulfur-replete medium or exposed to sulfur deprivation for 2 h prior to the RNA isolation. As shown in Figure 5A, the *SAC1* cDNA hybridizes with a 3.5 kb transcript that is present in both starved and unstarved wild-type cells. These results indicate that the isolated cDNA is full-length or nearly full-length and that the *SAC1* gene is active in both nutrient-replete and sulfur-starved cells. The presence of the *SAC1* transcript in non-starved cells is consistent with the possible role of the Sac1 polypeptide in the detection of sulfur limitation by *Chlamydomonas* or in the regulation of the acclimation response. In contrast to what was observed for wild-type cells, the *SAC1* cDNA hybridized to three distinct transcripts from the *sac1* mutant (Figure 5B). One of these transcripts is larger and two are smaller than the transcript that is present in wild-type cells. These transcripts are probably the result of fusions of *SAC1* and the sequences introduced during mutatgenesis.

Sequence analysis of the *SAC1* cDNA (pJD236) reveals that it has a 517 bp 5′ untranslated region (UTR), an open reading frame of 1755 bp and a 3′ UTR of 1129 bp. Partial sequence analysis of the *SAC1* genomic clone (pJD160) indicates that it is collinear with the cDNA except for an additional 65 bases at the 5′ end of the genomic clone and the presence of multiple introns. Furthermore, the cDNA contains ~400 bp of the 3′ UTR that has been truncated in the genomic clone. The truncated

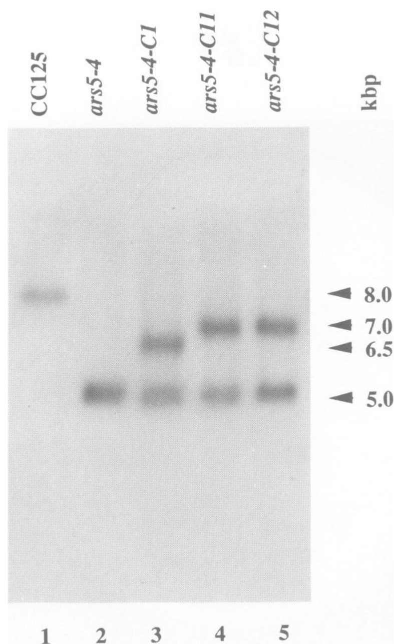


Fig. 4. DNA gel blot analysis of wild-type, *sac1* and complemented strains. DNA from CC125 (wild-type), *ars5-4* (*sac1*) and *ars5-4-C1*, *ars5-4-C11*, and *ars5-4-C12* (three complemented *sac1* strains) was isolated, digested with *Sal*I, separated by agarose gel electrophoresis, transferred onto nitrocellulose and hybridized with the 6.5 kbp *Sal*I insert from pJD160.

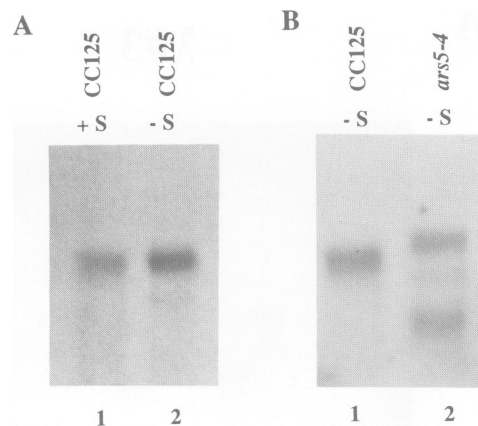


Fig. 5. RNA gel blot analysis of the *SAC1* transcript in wild-type and the *sac1* mutant. (**A**) Poly(A) purified RNA (4 μg per lane) isolated from CC125 grown in +S (lane 1) or –S medium for 2 h (lane 2) was separated by electrophoresis, transferred to nitrocellulose and hybridized with the *SAC1* cDNA (pJD236 insert). The transcript size is 3.5 kb. (**B**) Poly(A) purified RNA (4 μg per lane) isolated from both CC125 (lane 1) and *ars5-4* (lane 2) incubated in –S medium for 2 h was separated by electrophoresis, transferred to nitrocellulose and hybridized with the *SAC1* cDNA.

3′ end of *SAC1* in pJD160 has no apparent effect on the ability of this plasmid to complement the mutant phenotype. As deduced from the nucleotide sequence, the Sac1 polypeptide contains 585 amino acids (Figure 6A) and has a predicted molecular mass of 62.3 kDa. The polypeptide has five hydrophobic domains within the first half of the protein as well as a hydrophobic C-terminus (Figure 6B). While we were unable to find similarities to proteins of known function, we did detect a similarity between the deduced amino acid sequence of *SAC1* and orf f561 of *E.coli* (Allen *et al.*, 1992). orf f561 is located immediately downstream of the *hslAB* opreron (Allen *et al.*, 1992) which encodes two small heat shock proteins, and is within 5 kbp of an open reading frame with similarity to arylsulfatase (Burland *et al.*, 1993). Although still untested, orf f561 may have a role in regulating the expression of these genes.

Phenotypic analysis of the complemented strains

To confirm that all of the phenotypes displayed by the *sac1* mutants are caused by a lesion in *SAC1*, we compared Ars protein levels, *ARS* mRNA accumulation, accumulation of periplasmic proteins, sulfate uptake, cell viability and photosynthesis in wild-type, *sac1*, and the complemented *sac1* strains during sulfur-sufficient and sulfur-deficient growth. As shown in Figure 7, when wild-type cells (CC125) are transferred from a nutrient-replete medium to a medium lacking sulfur, Ars activity is detected after a 1–2 h lag period and the activity continues to increase for at least 24 h. No Ars activity is detected in the *sac1* strain (*ars5-4*) during this time. In the complemented strain (*ars5-4-C11*), the induction of Ars activity is similar to that of wild-type cells. Figure 8 shows that similar results are observed for the accumulation of *ARS* mRNA; while *ars5-4* shows no accumulation of *ARS* mRNA during sulfur deprivation, an increase in *ARS* mRNA, with kinetics that are similar to those seen in CC125, is observed in *ars5-4-C11*. Neither wild-type cells nor the complemented strain exhibit *ARS* mRNA accumulation in medium containing sulfur (data not shown).

We also compared sulfate transport in the complemented strain with that in wild-type and *sac1* mutant strains. The characteristics of sulfate transport in CC125, *ars5-4* and *ars5-4-C11* grown in sulfur-sufficient medium are essentially identical. As shown in Table III, upon sulfur deprivation of CC125 the $K_{1/2}$ for sulfate transport decreases by 5-fold while the V_{max} increases 10-fold (Yildiz *et al.*, 1994). However, in *ars5-4*, sulfate transport is not induced to the same extent as in wild-type cells; the transport system of the mutant has a higher affinity for the substrate during sulfur-limited growth (like wild-type cells) but the Vmax for sulfate only increases to a third of the level observed in wild-type cells. In *ars5-4-C11*, sulfur deprivation causes a decrease in the $K_{1/2}$ and an increase in the V_{max} comparable with that of CC125 (Table III).

When wild-type cells are starved for sulfur they accumulate several periplasmic proteins that are not present in cells grown in sulfur-replete medium. We compared the periplasmic proteins present in the wild-type, *sac1* and complemented *sac1* strains grown in sulfur-sufficient and sulfur-deficient medium. Many of the proteins that accumulate in sulfur-starved wild-type cells (CC2267) (marked by arrowheads in Figure 9) do not accumulate in the *sac1* strain (*ars5-4*). These sulfur-stress induced proteins are present in the complemented strain (*ars5-4-C11*). A notable difference in the protein profile of sulfur-starved CC2267 and *ars5-4-C11* cells is the presence of a 90 kDa protein in *ars5-4-C11* cells that is not present in CC2267. This difference is due to the genetic backgrounds of these strains. The original mutant, *ars5-1*, was isolated from mutagenized CC425; sulfur-starved CC425 accumulates this protein during sulfur

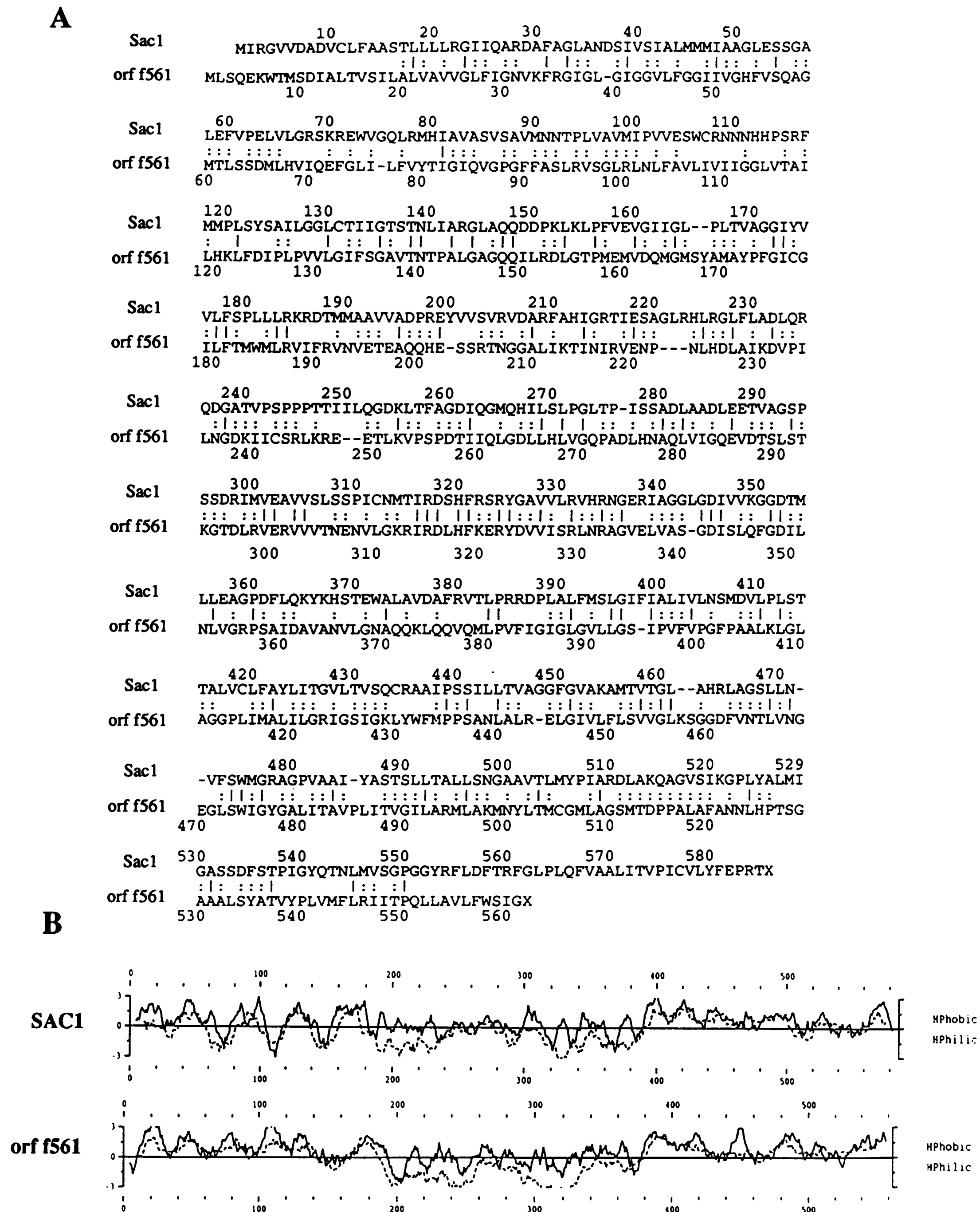


Fig. 6. Sequence comparison of the Sac1 polypeptide and orf f561. (**A**) The deduced amino acid sequence of Sac1 and orf f561 (an open reading frame of unknown function in *E.coli*) were aligned using the FASTA sequence comparison. The two sequences are 18% identical and 68% similar over the entire length of the sequences. (**B**) The solid line is a plot of the Kite–Doolittle hydropathy index of the Sac1 and orf f561 sequences using a window of nine residues for the calculation. The broken line is a plot of the Goldman, Engleman and Steitz index of Sac1 and orf f561 using a window of 20 amino acids for the calculation. The GenBank accession number for the SAC1 sequence is U47541.

stress. Another difference is the 40 kDa protein present in CC2267 grown on complete medium but not seen in either *ars5-4* or *ars5-4-C11*. This is also probably due to the difference in genetic background of these strains. We used CC2267 as the wild-type control for the analysis of periplasmic proteins because it was crossed with *ars5-1* to generate the *sac1 nit1* double mutant (*ars5-4*) that could be co-transformed with the *NIT1* gene (for complementation). Since CC425 and CC2267 are not isogenic, the progeny of this cross have a genetic background that is different from that of either parent, and the periplasmic proteins that accumulate are slightly different.

We have compared viability and photosynthetic activity in the wild-type, *sac1* mutant and complemented strains during sulfur deprivation. While the *sac1* mutant (*ars5-4*) dies within 2–3 days of sulfur deprivation, the complemented strain (*ars5-4-C11*) remains viable for >4 days, just like wild-type cells (CC125) (Figure 1A). Additionally, the complemented strain downregulates photosynthesis when sulfur-starved, while the mutant does not (Figure 2).

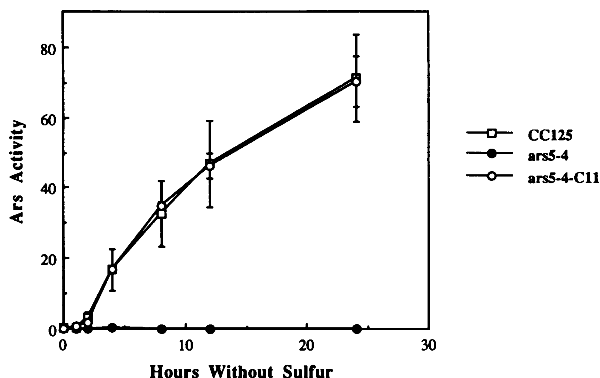


Fig. 7. Ars activity in sulfur-starved wild-type, *sac1* mutant and complemented *sac1* strains. Mid-logarithmic phase cultures (2–7×10^6 cells/ml) of CC125 (wild-type) (□), *ars5-4* (*sac1*) (●) and *ars5-4-C11* (complemented *sac1*) (○) were washed twice and resuspended in –S medium (2–4×10^6 cells/ml). Ars activity was measured using the chromogenic substrate *p*-nitrophenyl sulfate. The activity is expressed as μg of *p*-nitrophenol produced by 10^5 cells in 1 h. The data are averages of at least three experiments and the error bars represent the standard error of the mean.

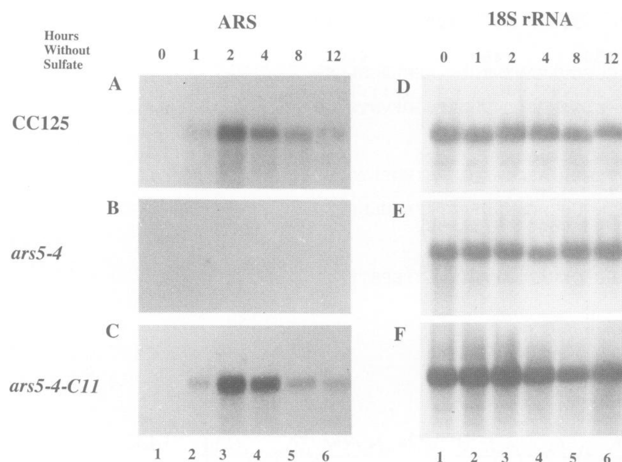


Fig. 8. *ARS* transcript accumulation in wild-type, *sac1* and complemented *sac1* strains. RNA isolated from CC125 (wild-type), *ars5-4* (*sac1*) and *ars5-4-C11* (complemented *sac1*) cells incubated in –S medium for 0, 1, 2, 4, 8 and 12 h. (**A**) Total RNA (10 μg) from each sample was separated by electrophoresis, blotted onto nitrocellulose and hybidized with the *ARS* cDNA. (**B**) To confirm that similar levels of RNA were loaded in each lane, the blots were stripped and hybridized with DNA for the 18S rRNA (plasmid P-92, obtained from the *Chlamydomonas* Genetics Center).

Table III. Characteristics of the sulfate transport system in wild-type *sac1* and a complemented *sac1* strain

Strain	+S		–S	
	V_{max}[a]	$K_{1/2}$[b]	V_{max}[a]	$K_{1/2}$[b]
CC125	27.5 (8.6)	16.8 (10.8)	356 (70)	2.11 (0.98)
ars5-4	18.3 (8.0)	10.0 (5.8)	129 (39)	2.17 (1.49)
ars5-4-C11	50.8(18.1)	7.1 (5.2)	391 (106)	2.26 (1.34)

Values are averages of at least three experiments. Standard errors are indicated in parentheses.
[a]Measured in fmol/s/10^5 cells.
[b]Measured in micromolar concentration.

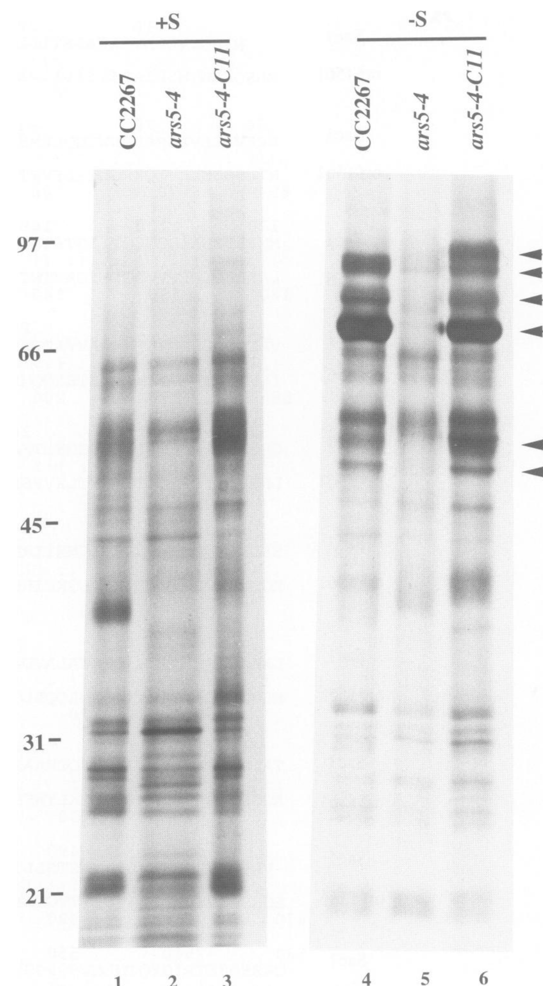


Fig. 9. Periplasmic proteins of wild-type, *sac1* mutant and complemented *sac1* strains grown in +S and –S media. Periplasmic proteins isolated from CC2267 (wild-type) (lanes 1 and 4), *ars5-4* (*sac1*) (lanes 2 and 5) and *ars5-4-C11* (complemented *sac1*) (lanes 3 and 6) were separated by SDS–PAGE and silver-stained (Davies *et al.*, 1994). Arrowheads designate proteins induced during sulfur deprivation; they are present in wild-type and complemented *sac1*, but not in the *sac1* mutant.

Together these results demonstrate that a functional *SAC1* gene is required for many of the responses that constitute the normal acclimation program of *Chlamydomonas* to sulfur deprivation. These responses include an elevation of *ARS* mRNA levels and Ars activity, an increase in sulfate transport and the accumulation of periplasmic proteins that are specific to sulfur-stressed cells. In addition, *SAC1* is required for *Chlamydomonas* to downregulate photosynthesis and withstand extended periods of sulfur limitation, making it a critical component of the acclimation machinery.

Discussion

We have used the presence of Ars activity as an indicator of sulfur deprivation in *Chlamydomonas* in order to screen for mutants that are unable to acclimate to sulfur-limited growth. One of these mutants is defective in the *SAC1* gene and is aberrant in many of its responses to sulfur limitation (Davies *et al.*, 1994). We show here that the

SAC1 gene product is required for survival of light grown, sulfur-deprived *Chlamydomonas* cells. While the *sac1* mutant dies during sulfur stress in the light, it survives sulfur deprivation in the dark and in the light in the presence of DCMU. Furthermore, we have demonstrated that while photosynthesis stops in sulfur-starved wild-type cells, it continues in the *sac1* mutant. Hence, to survive extended periods of sulfur starvation, the cells must decrease photosynthesis.

Harsh environmental conditions, such as nutrient limitation, low external temperatures and reduced water availability are known to cause a reduction in photosynthetic activity (Demmig-Adams and Adams, 1992). Nutrient limitation in particular can severely depress photosynthetic efficiency. Nitrogen deprivation causes a decrease in light-saturated CO_2 assimilation (Ferrar and Osmond, 1986; Saux *et al.*, 1987; Khamis *et al.*, 1990) and O_2 evolution (Henley *et al.*, 1991; Collier *et al.*, 1994), a reduction in the level of chlorophyll and fewer light harvesting complexes (Plumley and Schmidt, 1989; Peltier and Schmidt, 1991; Collier and Grossman, 1992), as well as less efficient transfer of the absorbed excitation energy from the light harvesting complex to photosystem II (Saux *et al.*, 1987; Collier *et al.*, 1994). Starvation of *Chlamydomonas* or cyanobacteria for sulfur also causes a decline in oxygen evolution (Figure 2; Collier *et al.*, 1994). In cyanobacteria, this decrease is correlated with a decline in photosystem II activity (Collier *et al.*, 1994). Sulfur limitation may also affect Calvin cycle enzymes such as RuBP carboxylase, which is actively degraded in sulfur-deprived *Lemna* (Ferreira and Teixeira, 1992).

Furthermore, during harsh environmental conditions photosynthetic organisms may absorb significantly more energy than they can use for photosynthesis. This excess excitation can lead to the production of active oxygen species through the donation of electrons from the photosynthetic electron transport chain directly to oxygen (Asada, 1994). Some of this excess light energy can be dissipated within the light harvesting complex as heat, thus lowering the potential for generating active oxygen species (Demmig-Adams and Adams, 1992). Thermal dissipation of energy is thought to be mediated, at least in part, by the carotenoid zeaxanthin (Khamis *et al.*, 1990; Björkman and Demmig-Adams, 1994). Sulfur-stressed *Dunaliella* cells accumulate high levels of zeaxanthin (Levy *et al.*, 1993), suggesting that more energy is dissipated as heat during sulfur limitation.

From the limited information available, nutrient-deprived cells appear unable to maintain high photosynthetic efficiencies, display decreased levels of light harvesting complexes and increased dissipation of excitation energy as heat. These changes appear to be part of a program of responses triggered by nutrient limitation. Recent measurements suggest that sulfur-deprived wild-type cells of *Chlamydomonas* exhibit reduced photosynthesis because of a block in photosynthetic electron transport (our unpublished data). Premature death of the *sac1* mutant appears to reflect the inability of this strain to modify photosynthetic electron flow during sulfur deprivation. Nutrient-starved cells unable to stop photosynthetic electron transport may produce high levels of reactive oxygen radicals, or generate highly reduced photosynthetic electron carriers which could cause aberrant regulation of metabolic processes; either of these situations could lead to cell death.

Our results demonstrate that the *SAC1* gene is necessary for cells to survive sulfur limitation. The only other genes known to be required for surviving nutrient limitation are the *BCY1* and *PDE2* genes of yeast. However, these genes appear to function in fundamentally different ways. The *BCY1* gene product, a negative regulator of cAMP-dependent protein kinase, and PDE2, a cAMP phosphodiesterase, are necessary to prevent cell cycle progression during nutrient stress (Toda *et al.*, 1987; Wilson and Tatchell, 1988), while the *SAC1* gene product is required for many responses that accompany the acclimation of cells to sulfur limitation; the response crucial for survival appears to be the downregulation of photosynthesis.

The sulfur-responsive signal transduction pathway in *Chlamydomonas* appears significantly different from that of *Neurospora* and other fungi. In *Neurospora* the *CYS3* gene product is the only positive acting factor known to affect expression of *ARS* and other sulfur-regulated proteins. *CYS3* encodes a bZIP DNA binding protein (Fu and Marzluf, 1990; Paietta, 1992) that is both necessary (Paietta, 1989) and sufficient (Paietta, 1990) to induce *ARS* transcription and the expression of other sulfur-regulated proteins (Marzluf, 1968, 1970b; Metzenberg and Ahlgren, 1970). The *CYS3* gene is negatively regulated by the *SCON1* and *SCON2* gene products and positively regulated by its own gene product (Paietta, 1990). The *MET4* gene product of yeast appears to be functionally similar to the *CYS3* gene product (Thomas *et al.*, 1992). In *Chlamydomonas*, we have identified at least two positive acting genes, *SAC1* and *SAC2* (Davies *et al.*, 1994), that regulate expression of Ars and other proteins regulated by the sulfur status of the environment. The *Chlamydomonas* *SAC1* gene product displays no similarity with bZIP DNA binding proteins, *SAC1* transcript levels are not controlled by sulfur availability and the negatively acting *SAC3* gene product does not appear to regulate the function of the *SAC1/SAC2* genes (Davies *et al.*, 1994). Furthermore, no *SAC1*-like gene has been implicated in the regulation of the sulfur deprivation response in fungi.

The *SAC1* gene product may sense the sulfur status of the cell or link signal perception to cellular responses. These possible functions are inferred from the observations that essentially all of the physiological responses to sulfur deprivation that we have measured are altered in strains defective in *SAC1*, and as expected for a protein that senses the sulfur status of the cell, the *SAC1* transcript (and most likely the Sac1 protein) is present in cells grown in both sulfur-replete and sulfur-deficient medium. The protein product, deduced from the sequence of the *SAC1* cDNA, has no similarity to proteins of known function, although there is significant similarity to a protein encoded by orf f561 of *E.coli* (Burland *et al.*, 1993). The orf f561 sequence is immediately downstream of an operon encoding two small heat shock proteins, designated *hslAB*, (Allen *et al.*, 1992) and within 5 kbp of an open reading frame that encodes a putative arylsulfatase (Burland *et al.*, 1993). The transcription of arylsulfatase genes in numerous organisms is activated in response to sulfur limitation (Adachi *et al.*, 1974; Apte *et al.*, 1974; Niedermeyer *et al.*, 1987; de Hostos *et al.*, 1989; Paietta, 1989; Hallmann and Sumper, 1994). The *hslAB* gene products are associated

with inclusion bodies which form under sub-optimal growth conditions, such as sulfur limitation. Although speculative at this point, the *E.coli hslAB* and *ars* genes may become active during sulfur-limited growth and be regulated by common factors, such as the protein product of the closely linked orf f561.

Materials and methods

Cell growth and mating

Cells were grown in nutrient-replete or sulfur-deficient Tris acetate phosphate liquid or solid medium at 100 µmol/m^2/s as previously described (Davies *et al.*, 1994). Mating of the various strains was performed according to the protocol of Harris (1989).

Genomic library construction

Ten micrograms of DNA from a *sac1* strain (*ars5-1*) were partially digested with *Sau*3AI (1.5 U for 1 h). The digested DNA was treated with calf intestinal phosphatase (CIP) for 15 min at 37°C. After heating the sample for 15 min at 70°C to denature the CIP, the reaction mixture was extracted with phenol followed by chloroform and the DNA was precipitated and then washed with 70% ethanol. The precipitated DNA was dried and resuspended in TE. One half microgram of the partially digested genomic DNA was ligated to 1.0 µg of λEMBL3 that had been digested with *Bam*HI. The ligated material was packaged using Gigapack II (Stratagene, La Jolla, CA). A total of 1 000 000 recombinant phage were recovered. 100 000 phage from the *ars5-1* recombinant library were screened for hybridization to both *ARG7* and *ARS2*, as described by Maniatis *et al.* (1989). DNA from a positive λ phage was isolated and mapped. A 5 kbp *Sal*I fragment from a region of the insert that did not hybridize to either *ARG7* or *ARS2* was used to screen 100 000 phage of a genomic library prepared from wild-type *Chlamydomonas* DNA (Davies *et al.*, 1992).

Chlamydomonas transformation

The *sac1 nit1* (nitrate reductase) double mutant, designated *ars5-4*, was grown in SGII medium (Kindle, 1990) to mid-logarithmic phase (2–5×10^6 cells/ml). Cells were collected from 250 ml of culture by centrifugation (5000 *g* for 5 min), resuspended in 5 ml of SGII/NO_3^- medium (SGII medium lacking ammonia but containing 3 mM nitrate) and incubated under standard growth conditions for 3 h (Davies *et al.*, 1994). The cells were incubated with autolysin, prepared according to Harris (1989) for 45 min to remove the cell walls. Three hundred microlitres of the treated cells were mixed with 100 µl of 20% PEG and 300 mg of acid washed, baked glass beads (Thomas Scientific, Philadelphia, PA), 2 µg pMN24 and 2 µg of the DNA to be co-transformed into the cells. The mixture was vortexed for 15 s, the glass beads allowed to settle and the cell suspension above the settled beads plated onto solid SGII/NO_3^- medium (Kindle, 1990). After 5–7 days the transformants were detected as discrete colonies.

Nucleic acid isolation, manipulation and construction of a cDNA library

The isolation of total nucleic acids and DNA and RNA gel blot procedures were performed according to Davies *et al.* (1992). Poly(A) RNA was selected by oligo(dT) cellulose chromatography according to the protocol of Maniatis *et al.* (1982).

For preparing the cDNA library, total RNA was isolated from wild-type cells (strain CC125) that had been starved for sulfur for 1, 2 and 4 h. One and a half milligrams of RNA from each sample were pooled and poly(A) RNA purified (Maniatis *et al.*, 1982). Five micrograms of poly(A) RNA were mixed with 2.5 µg of the oligonucleotide GCCACTCGAG$(dT)_{30}$ heated to 70°C for 10 min and then quickly chilled on ice. The annealing reaction was performed at 50°C for 2 min in the Superscript II reverse transcriptase buffer (Gibco, BRL) containing 10 mM DTT, 1 mM dATP, dGTP and dTTP, 1 mM methyl dCTP, and 40 U RNAse block (Stratagene, La Jolla, CA). First-strand synthesis, catalyzed by the addition of 700 U of Superscript II reverse transcriptase, was for 1 h at 50°C. Second-strand synthesis, addition of oligonucleotide linkers, ligation of the double stranded cDNA into λZAP and packaging of the phage DNA using Gigapack II were performed as suggested by the manufacturer (Stratagene, La Jolla, CA). The primary recombinant library contained 4.3×10^6 recombinant phage and was amplified according to the manufacturer's instructions.

Ars activity, sulfate uptake and the isolation of periplasmic proteins

Ars activity was measured as described by de Hostos *et al.* (1988) and Davies *et al.* (1994). Sulfate uptake experiments were performed as in Yildiz *et al.* (1994). Periplasmic proteins were isolated from the culture medium and resolved by SDS–PAGE as previously described (Davies *et al.*, 1994).

Oxygen evolution

Oxygen evolution was measured using a Hansatech DW2-2 Clark-type oxygen electrode. The culture was maintained at 27°C by a circulating waterbath and illuminated at 400 µmol/m^2/s by a fiberoptic system.

Cell viability

Cell viability was monitored by suspending cells in 0.025% phenosafranin, 0.025% methylene blue, 5 mM potassium phosphate and 10% ethanol. Live cells remained green while dead cells stained blue. Cells were counted in a hemocytometer.

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