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Mutants of *Chlamydomonas* with Aberrant Responses to Sulfur Deprivation

John P. Davies,[1] Fitnat Yildiz, and Arthur R. Grossman

Carnegie Institution of Washington, Department of Plant Biology, 290 Panama Street, Stanford, California 94305

In the absence of sulfur, *Chlamydomonas reinhardtii*, a unicellular green alga, increases its rate of sulfate import and synthesizes several periplasmic proteins, including an arylsulfatase (Ars). These changes appear to help cells acclimate to a sulfur-deficient environment. The elevated rate of sulfate import results from an increase in the capacity and affinity of the transport system for sulfate. The synthesis of Ars, a periplasmic enzyme that cleaves sulfate from aromatic compounds, enables cells to use these molecules as a source of sulfur when free sulfate is not available. To characterize the ways in which *C. reinhardtii* perceives changes in the sulfur status of the environment and regulates its responses to these changes, we mutagenized cells and isolated strains exhibiting aberrant accumulation of Ars activity. These mutants were characterized for Ars activity, *ars* mRNA accumulation, periplasmic protein accumulation, and sulfate transport activity when grown in both sulfur-sufficient and sulfur-deficient conditions. All of the mutants exhibited pleiotropic effects with respect to several of these responses. Strains harboring double mutant combinations were constructed and characterized for Ars activity and *ars* mRNA accumulation. From the mutant phenotypes, we inferred that both positive and negative regulatory elements were involved in the acclimation process. Both the epistatic relationships among the mutations and the effects of the lesions on the responses of *C. reinhardtii* to sulfur limitation distinguished these mutants from similar mutants in *Neurospora crassa*.

INTRODUCTION

The responses of microorganisms to sulfur-limited (−S) growth have been investigated in the prokaryotes *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella aerogenes*, and *Synechococcus* sp strain PCC 7942 and the eukaryotes *Neurospora crassa*, *Aspergillus nidulans*, *Saccharomyces cerevisiae*, and *Chlamydomonas reinhardtii*. Sulfate or the sulfur-containing amino acids cysteine and methionine are the preferred sources of sulfur for these organisms. When grown under −S conditions, these organisms respond by increasing sulfate transport and synthesizing the enzyme and transport systems necessary to exploit alternative sources of sulfur. Two mechanisms that can facilitate increased sulfate transport involve elevated synthesis of the constitutive sulfate permease and de novo synthesis of a high-affinity transport system. *E. coli*, *S. typhimurium*, and *Synechococcus* sp strain PCC 7942 have a single sulfate transport system that is synthesized at elevated levels during −S growth (Dreyfuss, 1964; Kredich, 1987; Green and Grossman, 1988; Laudenbach and Grossman, 1991). *N. crassa* has two sulfate permeases, one present primarily in conidia and the other in mycelia. When mycelia of *N. crassa* are grown in −S medium, synthesis of the mycelia-specific transporter increases (Marzluf, 1970; Ketter and Marzluf, 1988). *S. cerevisiae*, *A. nidulans*, and *C. reinhardtii* appear to have multiple sulfate transporters that are regulated by sulfur availability (Arst, 1968; Breton and Surdin-Kerjan, 1977; Yildiz et al., 1994).

Some organisms are able to exploit sources of sulfur other than sulfate, methionine, and cysteine by synthesizing specific sulfatases that cleave sulfate from organic compounds or by directly importing other sulfur-containing molecules. Arylsulfatase (Ars), an enzyme that cleaves sulfate from aromatic compounds, is synthesized during −S growth by *K. aerogenes* (Adachi et al., 1973), *A. nidulans* (Apte et al., 1974), *N. crassa* (Scott and Metzenberg, 1970), and *C. reinhardtii* (Lien and Schreiner, 1975; de Hostos et al., 1988). This periplasmic enzyme enables cells to use aromatic sulfates as a source of sulfur. In addition, *N. crassa*, when grown in −S conditions, can synthesize permeases that allow for import of choline sulfate and glucose-6-sulfate (Metzenberg and Parson, 1966).

The mechanisms by which cells regulate their responses to −S growth have been investigated in several organisms. In *E. coli* and *S. typhimurium*, the synthesis of proteins involved in sulfur acquisition and utilization is triggered by the accumulation of *O*-acetyl-L-serine, the immediate precursor of cysteine (Jones-Mortimer et al., 1968; Kredich, 1971). In *K. aerogenes*, transcription of *ars* is inhibited by sulfate or cysteine (Adachi et al., 1974; Murooka et al., 1990; Azakami et al., 1992). In *N. crassa*, the accumulation of Ars and the mycelial-specific

[1] To whom correspondence should be addressed.

permease is induced by growing mycelia in medium containing low levels of sulfur. The *cys-3* gene, which encodes a transcriptional activator (Marzluf and Metzenberg, 1968; Burton and Metzenberg, 1972; Paietta et al., 1987), is required for *N. crassa* to respond to −S growth. When starved for sulfur, *cys-3* mutants are unable to induce transcription of the genes encoding Ars and the mycelial sulfate permease. Furthermore, the expression of *cys-3* is governed by the negative regulatory factors encoded by the sulfur controller genes *scon-1* and *scon-2*. *scon-1* regulates the expression of *scon-2*, and *scon-2* regulates *cys-3* expression (Ketter and Marzluf, 1988; Paietta, 1989, 1990). In *S. cerevisiae*, the *MET4* gene serves an analogous function to the *cys-3* gene (Thomas et al., 1992).

Studies concerning the acclimation of photosynthetic eukaryotes to −S growth have been restricted to a few organisms (Ferrari and Renosto, 1972; Lass and Ullrich-Eberius, 1984; Rennenberg et al., 1988). We (de Hostos et al., 1988, 1989; Yildiz et al., 1994) and others (Lien and Schreiner, 1975; Schreiner et al., 1975; Sosa et al., 1978) have analyzed the responses of *C. reinhardtii* to growth in a −S environment. We are now exploring ways in which gene expression in *C. reinhardtii* is controlled during −S growth. Here, we describe three mutants with altered responses to −S conditions.

RESULTS

Mutagenesis and Mutant Isolation

One response of *C. reinhardtii* to −S is the synthesis of a periplasmic Ars (Lien and Schreiner, 1975; de Hostos et al., 1988). Ars activity can be easily detected in *C. reinhardtii* growing on −S agar medium supplemented with thiocyanate by spraying colonies with the chromogenic Ars substrate 5-bromo-4-chloro-3-indolyl sulfate (XSO_4) and monitoring for the appearance of a blue ring around the colonies. We used this characteristic to screen for mutants with aberrant responses to −S conditions.

C. reinhardtii CC425, a cell wall–deficient (*cw15*) arginine auxotroph (*arg2*), was transformed (Kindle, 1990) with the argininosuccinate lyase gene (the gene that complements the *arg2* lesion) (Debuchy et al., 1989). Transformants were selected as colonies that did not require exogenous arginine for growth. The introduced DNA integrated into the nuclear genome of *C. reinhardtii*, primarily at nonhomologous sites (Kindle et al., 1989; J.P. Davies, F. Yildiz, and A.R. Grossman, unpublished data). DNA gel blot analyses confirmed the presence of the introduced DNA (data not shown). Transformants contained two or more copies of the *arg2* gene: one copy represented the endogenous aberrant *arg2*, and the other(s) represented the introduced sequence. Using our protocol, most of the transformants received a single copy of the introduced DNA. The random integration of DNA into the genome may cause a mutation by disrupting a nuclear gene (Adam et al., 1993; Tam and Lefebvre, 1993). We can infer that the mutant phenotype is a consequence of the integration event by mating mutant and wild-type strains and correlating the aberrant phenotype in the progeny with the presence of the introduced DNA.

To isolate mutants aberrant in Ars expression, transformed colonies were screened for either the lack of Ars activity when grown on −S agar medium or constitutive expression of Ars in sulfur-sufficient medium (+S). To identify transformants that expressed little or no Ars, cells were grown for 4 days on −S agar medium containing 2 mM sodium thiocyanate. Under these conditions, wild-type cells used thiocyanate as a sulfur source for growth, but also expressed Ars. The colonies were then sprayed with XSO_4, and those expressing Ars activity formed a blue color around their periphery. Cells that showed little or no Ars activity were kept for further characterization. Transformants that were constitutively expressing Ars were identified by spraying colonies grown on +S medium with XSO_4. Ars-deficient mutants were observed at a frequency of ~1 in 1000, whereas those constitutively expressing Ars were observed at a frequency of ~1 in 5000.

The three mutant strains that we initially characterized, designated *sac1*, *sac2*, and *sac3* (sulfur-acclimation mutants), are shown in Figure 1. *sac1* had no Ars activity, and *sac2* exhibited low Ars activity when grown under −S conditions. Neither *sac1* nor *sac2* mutants exhibited Ars activity on +S medium. In contrast, *sac3* exhibited a high level of Ars activity when grown on −S medium and a low level of Ars activity when grown on +S medium. To determine whether the lesions were in genes located in the nucleus or an organelle, the mutants were crossed with the wild-type strain CC124 (*mt*−; in this study, CC124 and CC125 are considered wild-type strains; these strains differ only at the mating-type locus). The mutant phenotypes were inherited in a 2:2 ratio, indicating that all the lesions are in nuclear genes. To determine whether the mutations were linked, pairwise crosses were made. Tetratypes with the wild type, each of the single mutant, and double mutant phenotypes were obtained from each cross, indicating that the lesions were in different genes. Using DNA gel blot analyses, we determined that the endogenous *ars* gene was not interrupted in any of these strains (data not shown).

The mutants were originally isolated in a cell wall–deficient genetic background (*cw15*). However, because it was difficult to dissect viable progeny from tetrads of the cell wall–deficient strains, *sac* mutants with a wild-type cell wall were used in the genetic analyses. For comparative purposes, we measured Ars activity and *ars* mRNA accumulation in the cell wall plus and cell wall minus genetic backgrounds; the cell wall character did not alter Ars induction or *ars* mRNA accumulation in any of the strains (data not shown). The *sac* mutants in the cell wall plus genetic background were also used to measure sulfate transport because the cell wall–deficient strains were more susceptible to lysis during the assay procedure. Finally, because it is easy to isolate periplasmic proteins from cell wall–deficient strains, we used them to compare accumulation of Ars and other periplasmic proteins.

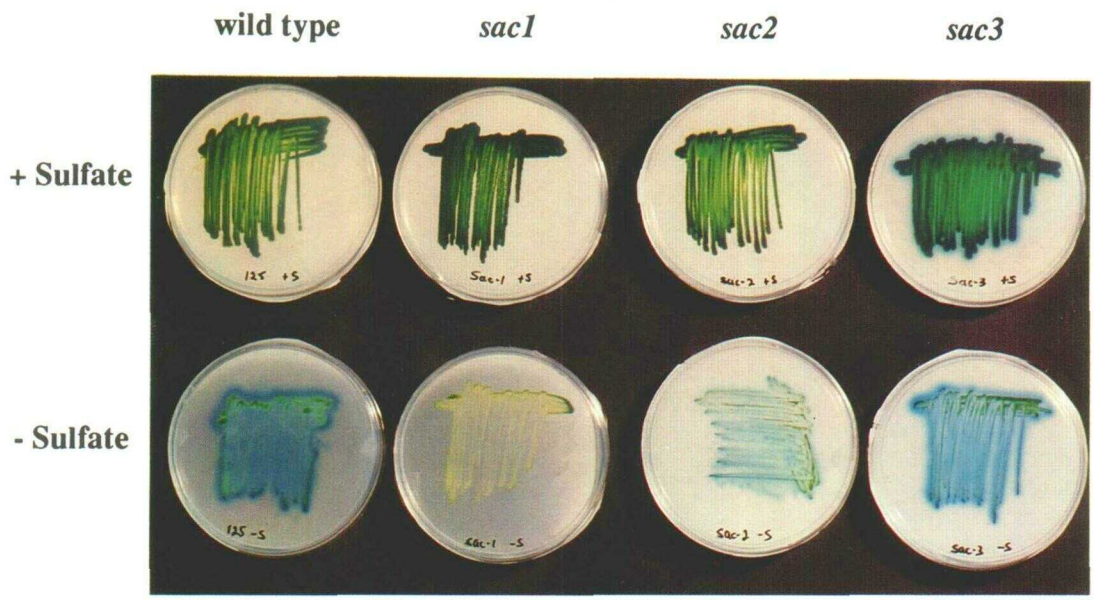

Figure 1. Accumulation of Ars Activity in the *sac* Mutants.

Cultures were spread on solid +S medium (+ sulfate) or −S medium supplemented with 2 mM thiocyanate (− sulfate), grown for 4 days, and sprayed with XSO_4. The color was allowed to develop for 10 hr.

Ars Activity

The levels of Ars activity were compared in the wild-type, *sac1*, *sac2*, and *sac3* mutant strains at various times after transferring the cells from +S to −S medium. Figure 2 presents data comparing Ars activity in the wild-type strain (CC125 *mt*+) and the *sac* mutants. In wild-type cells, Ars activity was detected 4 hr after transfer of cells to −S medium, and it continued to increase for 24 hr (72 units maximum). *sac1* mutants grown in −S medium exhibited no Ars activity, whereas *sac2* mutants exhibited very little (~4 units) after 24 hr under −S conditions. The *sac3* strain exhibited low Ars activity immediately after being washed with −S medium (7 units). Between 4 and 12 hr after transfer to −S medium, Ars activity in *sac3* mutants and the wild type increased identically, but after 12 hr, Ars activity in *sac3* no longer increased.

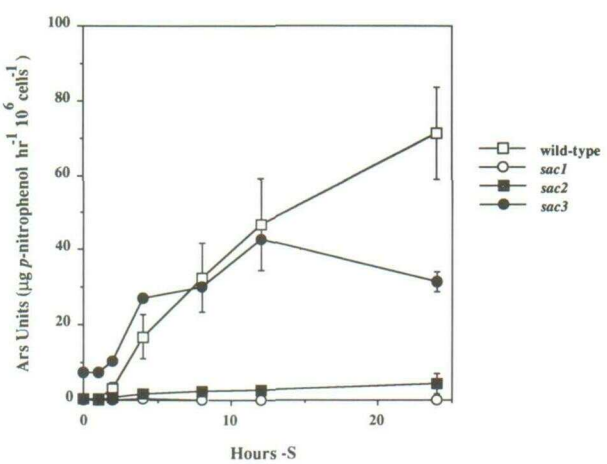

Figure 2. Accumulation of Ars Activity in the Wild Type and the *sac* Mutants Following Transfer to −S Medium.

Ars activity was measured using the chromogenic substrate *p*-nitrophenyl sulfate. The activity is expressed as micrograms of *p*-nitrophenol produced by 10^6 cells per 1 hr. The data presented are averages of three separate experiments, and the error bars represent the standard error of the mean. The strains tested were wild type (□), *sac1* (○), *sac2* (■), and *sac3* (●).

Ars Accumulation

To determine whether Ars was synthesized and processed correctly in the *sac* mutants, protein gel blot analyses were performed on periplasmic proteins. Figure 3 shows the accumulation of the Ars polypeptide in cells grown in +S (lanes

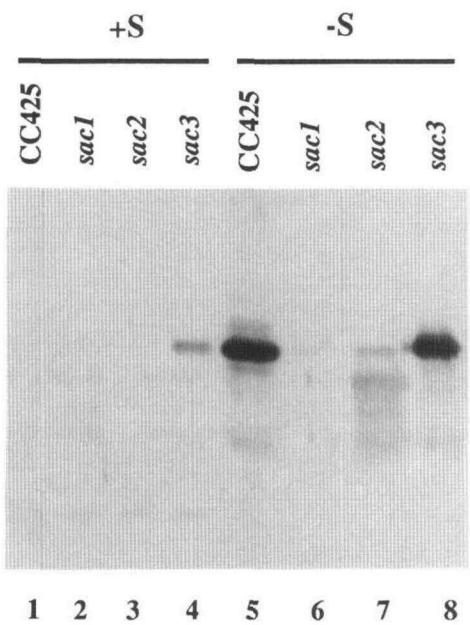

Figure 3. *Protein Gel Blots Showing Ars Accumulation in CC425 and the sac Mutants.*

Periplasmic proteins from cells grown in +S (lanes 1 to 4) and for 24 hr in −S media (lanes 5 to 8) were isolated, electrophoresed into polyacrylamide gels, transferred to nitrocellulose paper, reacted with Ars antiserum, and developed using the protein A–horseradish peroxidase system (Boehringer Mannheim). Proteins from CC425, and the *sac1*, *sac2*, and *sac3* mutants are in lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 8, respectively.

1 to 4) or −S medium (lanes 5 to 8) for 24 hr. The periplasmic proteins were isolated and fractionated by ammonium sulfate precipitation. Proteins that precipitated between 20 and 80% ammonium sulfate saturation (no Ars protein is detected among proteins precipitated in 20% ammonium sulfate) were separated by SDS-PAGE, transferred to nitrocellulose, and exposed to antiserum raised against Ars (de Hostos et al., 1989). No Ars protein was detected in the medium of CC425, *sac1*, or *sac2* cells grown in +S medium (Figure 3, lanes 1 to 3). However, Ars was detected in a *sac3* culture grown in +S medium (Figure 3, lane 4). This protein migrated identically with Ars from CC425 grown in −S medium (Figure 3, lane 5), indicating that both the synthesis and maturation of Ars occurred properly in *sac3* cells grown in +S medium.

After 24 hr of growth in −S medium, Ars was present in the CC425 culture (Figure 3, lane 5). In contrast, no Ars protein was detected in cultures of *sac1* (Figure 3, lane 6), and only a low level of Ars was detected in the *sac2* culture (Figure 3, lane 7). The Ars antibodies reacted with polypeptides from −S-grown *sac2* that migrated more rapidly than mature Ars. These polypeptides may have resulted from improper glycosylation and/or degradation of the Ars polypeptide. High levels of mature Ars accumulated in *sac3* cells grown in −S medium (Figure 3, lane 8). Comparison of Ars accumulation at specific intervals following the initiation of growth in −S medium using protein gel blot analysis (data not shown) correlated with measurements of Ars activity shown in Figure 2.

ars Transcript Accumulation

RNA gel blot analyses were performed to determine whether *sac1*, *sac2*, and *sac3* mutations caused altered *ars* transcript accumulation. Figure 4 compares *ars* transcripts from the wild type and *sac* mutants in a cell wall plus genetic background. *ars* mRNA was detected in wild-type cells 1 to 2 hr after the initiation of sulfur starvation; the transcript levels peaked at ~4 hr and decreased thereafter (Figure 4A). Because Ars activity increased up to 24 hr after the initiation of sulfur deprivation (Figure 2) and by 24 hr the transcript was barely detectable, we concluded that the Ars protein must be stable. In contrast to the wild type, *sac1* did not accumulate detectable levels of the *ars* transcript (Figure 4B) and *sac2* only accumulated a small amount. In *sac2*, *ars* transcript accumulation reached a maximum at ~2 hr after the initiation of −S conditions and was barely detectable after 4 hr (Figure 4C). In the *sac3* mutant, the temporal accumulation of *ars* mRNA was similar to the wild type, although it was more abundant after 24 hr in −S medium (Figure 4D).

Even though the Ars protein and enzymatic activity were detectable in *sac3* cells grown on +S medium, we could not always detect the transcript immediately after washing cells with −S medium. To determine whether the *ars* transcript was present in cells grown in +S medium, we isolated RNA from unwashed cells grown in +S medium and analyzed it for the presence of *ars* transcript. The wild-type, *sac1*, and *sac2* strains contained no detectable *ars* transcript when grown under +S conditions (Figure 4E, lanes 1 to 3), whereas *sac3* cells contained low levels of *ars* mRNA (Figure 4E, lane 4). A comparison of the levels of *ars* transcripts in total RNA from induced wild-type cells and uninduced *sac3* cells (Figure 4E, lanes 4 to 7) demonstrated that the transcript was present in the latter strain at a level of slightly less than 10% of that observed for fully induced wild-type cells. Because it was difficult to detect the *ars* transcript in *sac3* cells immediately after washing them with −S medium, the washing procedure probably inhibited *ars* transcription, and most of the transcript present initially was degraded.

Pleiotropic Effects of the *sac* Lesions

To investigate whether the *sac* lesions affected aspects of the cells response to −S growth other than Ars expression, we compared the accumulation of periplasmic proteins present in CC425 and the *sac* mutants in cells grown in both +S and −S media. Figure 5 shows that there were significant differences in periplasmic proteins isolated from cells grown in +S and −S media (compare lanes 1 and 5). Several new proteins

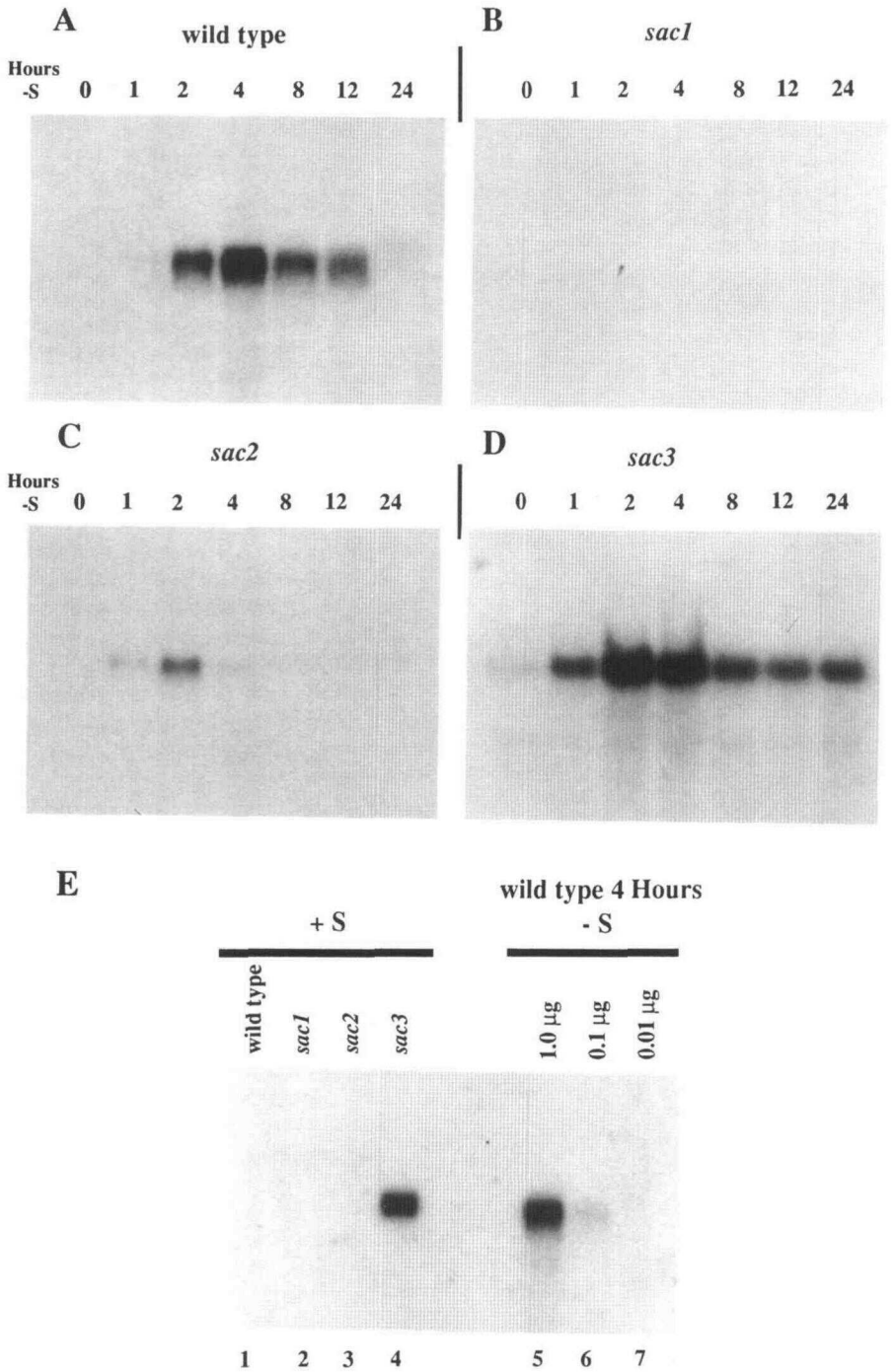

Figure 4. Accumulation of *ars* Transcripts during −S and +S Growth of Wild-Type and the *sac* Mutant Strains.

(A to **D)** Cells were grown in +S medium to midlogarithmic phase, washed twice with −S medium, and resuspended in −S medium. RNA was isolated at 0, 1, 2, 4, 8, 12, and 24 hr after the initiation of sulfur starvation. The RNA was separated by electrophoresis on 1% agarose gels, transferred to nitrocellulose paper, and hybridized with the *ars* cDNA. In **(A)**, RNA was from the wild-type strain (CC125), and in **(B)**, **(C)**, and **(D)**, RNA was from the mutant strains *sac1*, *sac2*, and *sac3*, respectively.

(E) RNA from mutant and wild-type cells grown in +S medium (lanes 1 to 4) and wild-type cells placed in −S medium for 4 hr (lanes 5 to 7). Ten micrograms of total RNA was loaded in each lane except in lanes 5 to 7, which contained a serial dilution (1.0, 0.1, and 0.01 µg) of wild-type RNA.

accumulated when the cells were deprived of sulfur (Figure 5, closed arrows), while other proteins that were normally present in cells grown in +S medium disappeared (Figure 5, open arrows). Other than Ars (Figure 5, lane 5, asterisk), the functions of these proteins are unknown. Although most periplasmic proteins that declined in abundance in CC425 cultures during −S growth also declined in the sac mutants, low levels of a 40-kD polypeptide not observed in −S-grown CC425 were observed in periplasmic proteins from *sac1* and *sac3* mutants grown in −S medium (Figure 5, lanes 6 and 8). Furthermore, in *sac1* and *sac2*, the periplasmic proteins that normally accumulated during growth of CC425 in −S medium were not detected (compare Figure 5, lanes 5 to 7). We demonstrated previously that Ars was present at low levels in *sac2* mutant cells grown in −S medium (Figure 3, lane 7); the other proteins induced during −S growth may also be present at levels

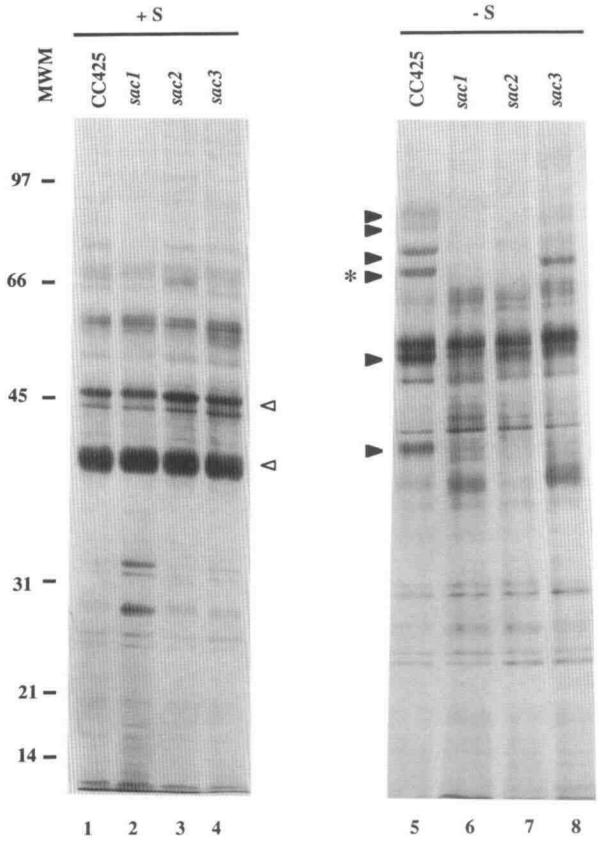

Figure 5. Periplasmic Proteins of CC425 and the *sac* Mutants Grown in +S and −S Media.

Periplasmic polypeptides from CC425 (lanes 1 and 5), *sac1* (lanes 2 and 6), *sac2* (lanes 3 and 7), and *sac3* (lanes 4 and 8) were isolated, separated by SDS-PAGE, and silver stained. Open arrowheads mark polypeptides present in cultures grown in +S but not −S medium. Closed arrowheads mark polypeptides that accumulated in cultures grown in −S but not +S medium. The arrowhead with the asterisk marks the Ars polypeptide. Molecular weight markers (MWM) are given at left in kilodaltons.

Table 1. Characteristics of the Sulfate Transport System in *sac* Mutants[a]

	+S Medium		−S Medium	
Strain	V_{max}[b]	$K_{1/2}$[c]	V_{max}[b]	$K_{1/2}$[c]
CC125	13.9 (2.2)	9.5 (1.7)	177.5 (28.8)	2.1 (0.9)
sac1	9.6 (3.3)	10.4 (5.4)	57.2 (9.6)	1.6 (0.6)
sac2	20.4 (3.5)	12.4 (0.7)	57.5 (20.7)	1.4 (0.6)
sac3	17.0 (5.4)	8.0 (0.8)	13.5 (6.6)	2.7 (1.3)

[a] Values are averages and standard errors of at least three experiments. Standard errors are indicated in parentheses.
[b] Measured in femtomoles per second per 10^5 cells.
[c] Measured in micromolars.

that are not readily visualized by protein staining methods. In *sac3* cells, Ars was the only sulfur stress–induced periplasmic protein that accumulated to high levels; the other induced periplasmic proteins were barely detectable.

To further assess the pleiotropic effects exhibited by the *sac* strains, we examined their sulfate transport activity. *C. reinhardtii* appears to have at least two systems for sulfate import. Both of these systems have a relatively high affinity for sulfate, although the permease expressed in cells deprived of sulfur has both a higher affinity and capacity than the permease present in cells grown in +S medium (Yildiz et al., 1994). To determine whether the *sac* mutants could synthesize the higher affinity transport system, we measured the $K_{1/2}$ (the sulfate concentration at which sulfate transport is half maximal) and V_{max} of sulfate transport in *sac* mutants grown for 6 hr in −S medium. Table 1 shows that the sulfate transport system of wild-type cells grown in +S medium had a V_{max} of ~14 fmol sec^{-1} 10^5 $cells^{-1}$ and a $K_{1/2}$ of ~10 μM. When grown in −S medium for 6 hr, the affinity for sulfate increased approximately fivefold ($K_{1/2}$ = 2 μM), and the capacity to import sulfate increased more than 10-fold (V_{max} = 175 fmol sec^{-1} 10^5 $cells^{-1}$) (Table 1). In all mutants grown in +S medium, the V_{max} values and $K_{1/2}$ concentrations of the transport system were similar to that of the wild type. In −S medium, all three mutants made the higher affinity sulfate transport system, but they were unable to fully induce transport (Table 1). The V_{max} values for sulfate transport in *sac1* and *sac2* were ~30% that of wild-type cells, whereas the V_{max} value for transport in the *sac3* mutant was ~10% that of the wild type.

Epistatic Relationships of the *sac* Mutants

To help define the role of the *sac* genes in the regulation of Ars expression, the epistatic relationships among the *sac1*, *sac2*, and *sac3* lesions were explored in double mutant combinations. To confirm the genotypes, we crossed each of the double mutants with the wild-type strain CC124 and obtained progeny exhibiting the phenotype of the wild-type strain, each of the single mutants, and the double mutant. Figure 6 and Table 2 show that cells containing a *sac3* lesion accumulated

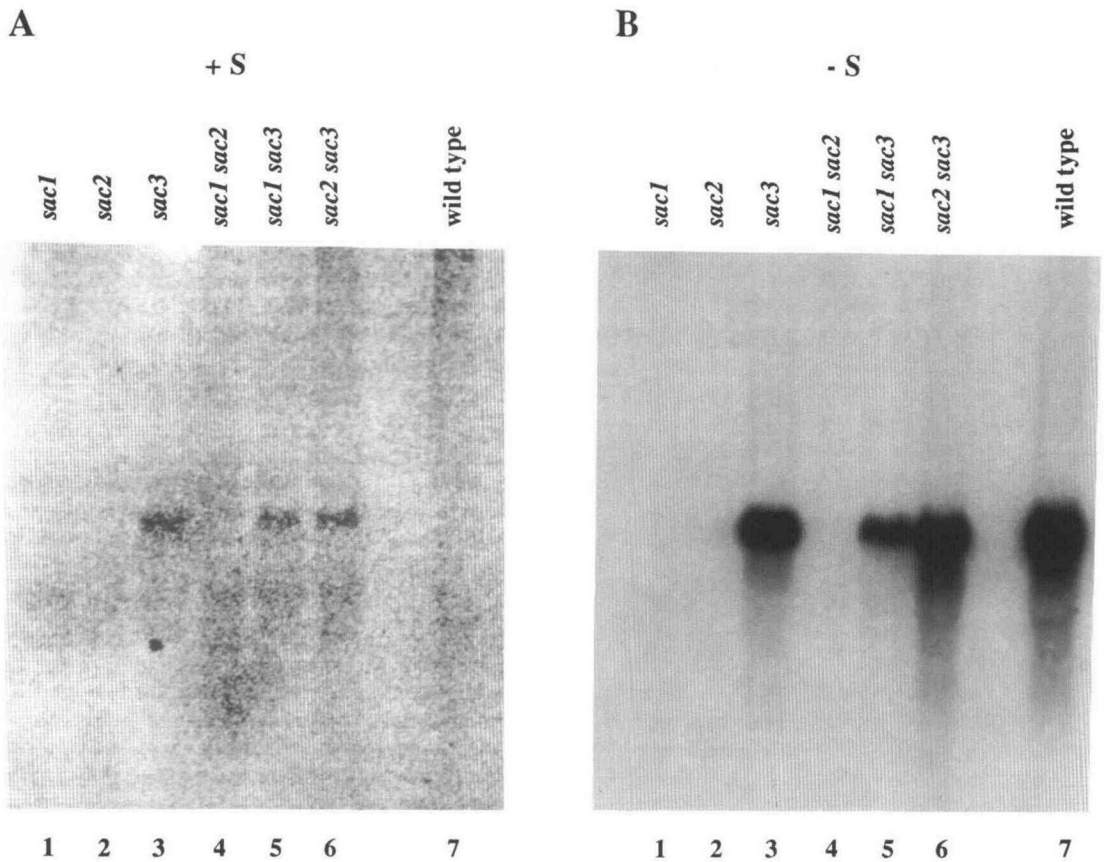

Figure 6. Detection of *ars* mRNA in Single and Double *sac* Mutants Grown in +S and −S Media for 4 Hr.

(A) RNA from cells grown in +S medium.
(B) RNA from cells grown in −S medium for 4 hr.
Ten micrograms of total RNA from *sac1* (lanes 1), *sac2* (lanes 2), *sac3* (lanes 3), *sac1 sac2* (lanes 4), *sac1 sac3* (lanes 5), *sac2 sac3* (lanes 6), and wild-type strain CC125 (lanes 7) was separated by electrophoresis on an agarose gel, transferred to nitrocellulose paper, and hybridized with the *ars* cDNA. The image in **(A)** was developed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

low levels of *ars* transcript (Figure 6A, lane 3) and exhibited low Ars activity (Table 2) in +S medium. During −S growth, *sac3* cells accumulated high levels of the *ars* transcript (Figures 4D and 6B, lane 3) and high levels of Ars activity (Figure 2 and Table 2; the final level of Ars activity was approximately one-half that of wild-type cells). The *sac1 sac3* and *sac2 sac3* double mutants also accumulated some *ars* transcripts (Figure 6A, lanes 5 and 6, respectively; 2% of fully induced wild-type cells) and exhibited low Ars activity in +S medium. However, the level of Ars activity in the double mutants was significantly lower than in the *sac3* single mutant (Table 2). In −S medium, the *ars* mRNA in the *sac1 sac3* and *sac2 sac3* double mutants accumulated to ~20% and 40% of that of wild-type cells, respectively (Figure 6B, lanes 5 and 6). Ars activity of *sac1*

Table 2. Ars Activity in the *sac* Mutants[a]

Strain	+S Medium	−S Medium
CC125	0.7 (0.5)	71.3 (12.4)
sac1	0.4 (0.3)	0.1 (0.1)
sac2	0.4 (0.2)	4.4 (2.7)
sac3	12.0 (4.3)	31.4 (3.5)
sac1 sac2	0.3 (0.2)	0.1 (0.1)
sac1 sac3	2.6 (0.6)	8.6 (2.5)
sac2 sac3	2.2 (0.8)	20.2 (5.4)

[a] Cells were grown in +S or −S medium for 24 hr. Ars activity is reported in units of micrograms of *p*-nitrophenol production by 10^6 cells per 1 hr. Values are averages and standard errors of at least three experiments. Standard errors are indicated in parentheses.

sac3 and *sac2 sac3* grown in −S medium increased similarly (Table 2).

In summary, the double mutants containing a *sac3* lesion exhibited a mixed phenotype; they accumulated *ars* mRNA and expressed Ars activity in both +S and −S media, but at lower levels than in the *sac3* single mutant. We were unable to detect *ars* transcript accumulation or Ars activity in the *sac1 sac2* double mutant grown in either +S or −S medium (Figure 6 and Table 2), indicating that the *sac1* lesion is probably epistatic to the *sac2* lesion. However, we cannot exclude the possibility that the double mutant has a mixed phenotype and that Ars activity and *ars* mRNA accumulation were below detectable levels. The phenotypes of the single and double mutants are beginning to provide clues about the roles of the *sac* gene products in the regulation of *ars* and the sulfur acclimation response.

DISCUSSION

During sulfur stress, *C. reinhardtii* accumulates Ars as well as other periplasmic polypeptides (Lien and Schreiner, 1975; de Hostos et al., 1988) and increases its rate of sulfate uptake (Yildiz et al., 1994). To examine how cells perceive the sulfur status of the environment and regulate their physiological responses to sulfur limitation, we generated and characterized mutants that exhibited aberrant levels of Ars activity. Critical aspects of the screen for these mutants were the use of a chromogenic assay for measuring altered Ars activity and the ability of wild-type cells to synthesize high levels of Ars when grown on agar medium devoid of sulfate but supplemented with thiocyanate as the sole sulfur source.

In this study, we have described three mutants, *sac1*, *sac2*, and *sac3*, that exhibited anomalous Ars expression. No Ars activity was observed in *sac1* grown in +S or −S medium. *sac2* exhibited no Ars expression in +S and low level expression in −S medium, whereas *sac3* exhibited low-level expression in +S and high-level expression in −S medium. The mutant phenotypes suggested that *ars* is controlled by both negative- and positive-acting factors. Positive-acting factors were inferred from the finding that none of the mutants fully induced Ars in sulfur-deficient medium. The constitutive low-level expression of Ars in the *sac3* mutant suggested the possibility that a negative-acting element may repress Ars expression in +S medium.

The *sac* mutants described here cause an alteration in a number of the responses that *C. reinhardtii* exhibits during −S growth. The *sac1*, *sac2*, and *sac3* strains were deficient in the accumulation of specific periplasmic polypeptides that normally appear during −S growth and did not fully induce the high-affinity sulfate transport system. This suggested that the genes altered in the *sac* strains had a general effect on the responses of cells to −S growth.

During sulfur stress, wild-type cells accumulated several periplasmic proteins. Whereas one of these proteins is Ars, the identities and functions of the others are not known. The *sac1* mutant did not accumulate detectable quantities of any of the periplasmic proteins that are normally induced during −S growth. In *sac2* and *sac3* mutants, only Ars was detected; *sac2* accumulated low levels of Ars, whereas in *sac3*, Ars activity accumulated to approximately one-half the level of that measured in wild-type cells. This suggested that all of the periplasmic proteins that accumulated during −S growth were regulated by some common elements.

The *sac* mutants also showed aberrant levels of sulfate transport. Wild-type *C. reinhardtii* cells exposed to −S medium increased their affinity for sulfate by approximately fivefold and their capacity to import sulfate by more than 10-fold when compared to cells grown in +S medium (Yildiz et al., 1994). The affinity of the sulfate transport system in *sac1*, *sac2*, and *sac3* increased to wild-type levels during the acclimation process, but the capacity for sulfate transport did not increase to the same extent as in wild-type cells. Hence, the capacity for sulfate uptake during −S growth was controlled by at least some of the same factors that control the expression of *ars*.

It is interesting that the *sac3* mutant, which was selected for constitutive accumulation of Ars during +S growth, accumulated Ars only to levels ~50% of that observed in wild-type cells, was unable to accumulate much of the other sulfur stress–induced periplasmic polypeptides, and did not develop high levels of sulfate transport during −S growth. These data suggest that the *sac3* gene product was not only important for repressing *ars* transcription during +S growth but was also required for the full induction of Ars, the accumulation of the other sulfur-regulated periplasmic proteins, and the increase in sulfate transport activities. Hence, under +S conditions the *sac3* gene product may act as a negative regulator, while under −S conditions it may serve as a positive regulator. Because *sac3* mutants accumulated Ars activity at the same rate as wild-type cells when initially transferred to −S medium, the Ars polypeptide was probably synthesized at the same rate in the two strains. However, because after 24 hr in −S medium *sac3* mutants had only half the Ars protein and activity of wild-type cells despite containing more *ars* mRNA, the stability of the Ars protein may have been altered.

To begin dissecting the functions of the *sac1*, *sac2*, and *sac3* gene products, we generated the double mutants *sac1 sac2*, *sac1 sac3*, and *sac2 sac3* and analyzed accumulation of *ars* mRNA and Ars activity during growth of these strains in both +S and −S media. All cells containing the *sac3* lesion accumulated *ars* mRNA and exhibited Ars activity in +S medium, but the double mutants exhibited lower *ars* mRNA accumulation and less Ars activity. In addition, both the *sac1 sac3* and *sac2 sac3* strains had lower levels of *ars* mRNA and less Ars activity than the *sac3* single mutant in −S medium. Thus, the lesion in *sac1* and *sac2* reduced, but did not completely block, expression of Ars in the *sac3* strain grown in either +S or −S medium. These complex phenotypes suggest that the *sac1* and *sac2* gene products may have functioned in parallel with the *sac3* gene product to regulate *ars* transcription or transcript accumulation. However, because *sac2* accumulated

appreciable quantities of an improperly processed or degraded form of Ars, it is possible that this mutant contains a lesion in the machinery for export and/or processing of proteins during sulfur stress. The inability to export Ars properly may cause the internal accumulation of Ars, which, in turn, could inhibit *ars* mRNA accumulation.

There are both similarities and differences between the mechanisms regulating −S-related changes in gene expression in *C. reinhardtii* and *N. crassa*. In both organisms, *ars* appears to be controlled by genes that exert positive and negative regulatory effects (Marzluf and Metzenberg, 1968; Metzenberg and Ahlgren, 1970; Burton and Metzenberg, 1972). In *N. crassa*, the positive regulator *cys-3* is a transcriptional activator (Marzluf and Metzenberg, 1968; Paietta, 1992) that may act directly on the *ars* promoter. Strains harboring *cys-3* lesions, when grown in −S conditions, do not express Ars or any other sulfur-regulated protein (Metzenberg and Parson, 1966; Marzluf and Metzenberg, 1968; Paietta, 1989). In contrast, cells with a lesion in the negative regulators *scon-1* and *scon-2* express Ars and other sulfur-regulated proteins in +S medium (Burton and Metzenberg, 1972; Fu et al., 1990; Paietta, 1990). In addition, a mutation in the positive regulator *cys-3* is epistatic to lesions in the negative regulator *scon-1* (Dietrich and Metzenberg, 1973; Paietta, 1990).

The phenotype of the *sac1* mutant of *C. reinhardtii* shares some similarities with that of the *cys-3* mutant of *N. crassa*. When starved for sulfur, *sac1* mutants did not exhibit Ars activity or accumulate *ars* mRNA. They also did not accumulate any of the other sulfur-regulated periplasmic proteins. However, unlike a *cys-3*–defective strain, −S-grown *sac1* mutants did accumulate moderate levels of an inducible, very high-affinity sulfate transport system. Similar to *scon-1* and *scon-2* mutants, *sac3* mutants accumulated *ars* transcripts and displayed Ars activity in +S medium (Burton and Metzenberg, 1972). However, the *scon-1* mutant exhibits increased sulfate permease activity during growth in +S medium (Burton and Metzenberg, 1972), whereas the *sac3* strain did not. Another difference between the sulfur acclimation systems of *N. crassa* and *C. reinhardtii* is in the epistatic relationships of the positive- and negative-acting regulators of Ars expression. In *N. crassa*, lesions in the positive regulator *cys-3* are clearly epistatic to lesions in the negative regulator *scon-1* (Dietrich and Metzenberg, 1973), whereas in *C. reinhardtii*, double mutant combinations of the putative positive-acting regulator *sac1* with the putative negative-acting regulator *sac3* had a mixed phenotype.

The mutants described here were isolated following transformation of *C. reinhardtii* with the *arg2* gene (Debuchy et al., 1989), and both the *sac1* and *sac3* lesions were tagged with the introduced DNA (data not shown). This strategy allowed us to clone the aberrant gene from at least one of these mutant strains, and this clone was used to isolate fragments of DNA from a wild-type genomic library that complemented the mutation (data not shown). The further generation and characterization of mutant strains coupled with the identification of the genes harboring the lesions will help to elucidate the molecular mechanisms that control both the perception and responses of *C. reinhardtii* to sulfur stress.

METHODS

Cell Culture

Cells were cultured in either sulfur-replete Tris-acetate-phosphate medium (+S medium) (Gorman and Levine, 1966) or Tris-acetate-phosphate medium lacking sulfur (−S medium). In −S medium, the sulfate anion was replaced with chloride. For sulfur deprivation experiments in liquid medium, the cells were grown to midlogarithmic phase in +S medium, washed twice with −S medium (3000*g* for 5 min), and resuspended in −S medium to a final density of 2 to 8 × 10^6 cells per mL. To grow cells on solid medium under −S conditions, the −S medium was supplemented with 2 mM sodium thiocyanate and 1% agar that was sulfate free. Under these conditions *Chlamydomonas reinhardtii* grows, using thiocyanate as a sulfur source, and expresses high levels of arylsulfatase (Ars) activity. Sulfate was removed from Nobel agar (U.S. Biochemical Corp.) by washing it twice with ethanol and six times with distilled water. The agar was dried by lyophilization.

Matings

The various strains were mated according to the protocol of Harris (1969).

Mutagenesis

The *C. reinhardtii* arginine auxotroph CC425 (*mt+ cw15 arg2*) was mutagenized by transformation with DNA containing the argininosuccinate lyase (*arg2*) gene (Debuchy et al., 1989). Transformations were performed as described previously (Davies et al., 1992) with plasmids containing an intact copy of the *arg2* gene. Cells (5 to 10 × 10^7) growing in midlogarithmic phase were collected, resuspended in 1 mL of +S medium supplemented with 50 μg/mL arginine and 5% polyethylene glycol (Molecular Biology Grade; Sigma Chemicals), and added to 300 mg of acid washed and baked glass beads. Two micrograms of linear pJD76 or pJD67 was added, and the mixture was vortexed at full speed. Five milliliters of +S medium supplemented with 50 μg/mL arginine was added, and the culture was placed on an illuminated (90 μE m^{-2} sec^{-1}) rotating platform for 16 to 20 hr. The cells were separated from the glass beads, pelleted by centrifugation (3 min at 3000*g*), washed twice with +S medium, resuspended in 100 μL of +S medium, and spread on solid +S medium. Approximately 300 colonies grew on each plate.

pJD76 is a pUC19 derivative containing the 5′ and 3′ regions of *ars* genomic sequences interrupted by the *arg2* gene (5′*ars*/*arg2*/3′*ars*) and was constructed for the purpose of interrupting the endogenous *ars* gene. It is composed of a 2-kb HindIII fragment from the 5′ end of *ars* (1 kb of the promoter and 1 kb of the transcribed region; de Hostos et al., 1989) fused to the *arg2* gene (Debuchy et al., 1989) and a 2-kb MluI-SacI fragment containing the 3′ end of *ars* (de Hostos et al., 1989). Prior to transformation, the DNA was linearized by digestion with EcoRI; linearization restricts the DNA at one site upstream of the 5′*ars* and

one site downstream of *3'ars* to release all of the *5'ars/arg2/3'ars* insert from pJD76. Transformation with this fragment generated the sulfur acclimation *sac1* mutant strain. pJD67 contains the *arg2* gene (Debuchy et al., 1989) in the vector pBluescript KS+ (Stratagene). This DNA was linearized by digesting the plasmid with HindIII, which cuts the DNA at a unique site within the multiple cloning region of the vector, and was introduced into *C. reinhardtii* to generate the sulfur acclimation *sac2* and *sac3* mutant strains.

Ars Activity Assay

Ars activity of colonies growing on solid medium was assayed by spraying the colonies with 300 to 500 μL of 10 mM 5-bromo-4-chloro-3-indolyl sulfate (XSO_4) in 0.1 M Tris-HCl, pH 7.5. Within 1 to 2 hr, a blue color formed around each colony synthesizing Ars. Ars activity in liquid cultures was assayed using *p*-nitrophenyl sulfate as the substrate as described by de Hostos et al. (1988), except that the reaction was incubated for 30 min at 27°C.

Purification of Periplasmic Proteins

Proteins were isolated from cells with the *cw15* lesion grown in +S medium to midlogarithmic phase, or after they had been transferred to −S medium for 24 hr. Five molars NaCl was added to the culture until the final concentration was 0.1 M NaCl. The culture was agitated for 10 min, and the cells were pelleted by centrifugation (2000*g* for 5 min). Ammonium sulfate was added to the supernatant to 20% saturation (14.1 g/100 mL), and the supernatant was stirred at 4°C for 1 hr. Precipitated proteins were pelleted by centrifugation for 15 min at 8000*g*. The supernatant was brought to 80% saturation with ammonium sulfate (66.5 g/100 mL), stirred at 4°C for 1 hr, and centrifuged as given above. The proteins that precipitated between 20 and 80% saturating ammonium sulfate were resuspended in 50 mM Tris-acetate, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, and 1 mM ε-amino-*n*-caproic acid. To reprecipitate these proteins in preparation for electrophoresis, trichloroacetic acid (100% w/v) was added to 10%. After 1 hr on ice, protein was collected by centrifugation at maximum speed in a microcentrifuge (Brinkmann Instruments) and resuspended by sonication in 75 μL of 0.1 M sodium carbonate, 0.1 M dithiothreitol. One-half volume of SDS-PAGE sample buffer (5% SDS, 30% sucrose, 0.1% bromophenol blue) was added to the sample prior to boiling for 1 min. Samples were frozen in liquid nitrogen and stored at −80°C. Electrophoresis of the samples was on a 8 to 15% polyacrylamide gradient gel using standard conditions (Laemmli, 1970). The proteins were fixed by incubating the gel in 20% methanol, 7% acetic acid, and then they were stained with silver (Porro et al., 1982).

Sulfate Uptake Analysis

Sulfate transport was measured as described by Yildiz et al. (1994). $K_{1/2}$ (the sulfate concentration at which sulfate transport is half maximal) and V_{max} values were calculated from a Lineweaver–Burke plot of the data.

Protein, DNA, and RNA Blot Analysis

Protein blot analysis was performed as described by de Hostos et al. (1988). Nucleic acids were purified as described by Davies et al. (1992) and quantitated by measuring the absorbance at 260 nm. DNA and RNA gel blot analyses were performed as described by Davies et al. (1992). Radiolabeled *ars* cDNA (de Hostos et al., 1989) was hybridized to RNA that had been transferred to nitrocellulose paper according to the methods of Davies et al. (1992).

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