

# Salt Induction of Fatty Acid Elongase and Membrane Lipid Modifications in the Extreme Halotolerant Alga *Dunaliella salina*<sup>1</sup>

Malkit Azachi<sup>2</sup>, Avi Sadka<sup>3</sup>, Morly Fisher<sup>4</sup>, Paulina Goldshlag, Irena Gokhman, and Ada Zamir\*

Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel (M.A., A.S., M.F., I.G., A.Z.); and Volcani Center, Bet Dagan 50250, Israel (P.G.)

In studies of the outstanding salt tolerance of the unicellular green alga *Dunaliella salina*, we isolated a cDNA for a salt-inducible mRNA encoding a protein homologous to plant  $\beta$ -ketoacyl-coenzyme A (CoA) synthases (Kcs). These microsomal enzymes catalyze the condensation of malonyl-CoA with acyl-CoA, the first and rate-limiting step in fatty acid elongation. Kcs activity, localized to a *D. salina* microsomal fraction, increased in cells transferred from 0.5 to 3.5 M NaCl, as did the level of the *kcs* mRNA. The function of the *kcs* gene product was directly demonstrated by the condensing activity exhibited by *Escherichia coli* cells expressing the *kcs* cDNA. The effect of salinity on *kcs* expression in *D. salina* suggested the possibility that salt adaptation entailed modifications in the fatty acid composition of algal membranes. Lipid analyses indicated that microsomes, but not plasma membranes or thylakoids, from cells grown in 3.5 M NaCl contained a considerably higher ratio of C18 (mostly unsaturated) to C16 (mostly saturated) fatty acids compared with cells grown in 0.5 M salt. Thus, the salt-inducible Kcs, jointly with fatty acid desaturases, may play a role in adapting intracellular membrane compartments to function in the high internal glycerol concentrations balancing the external osmotic pressure.

Unicellular green algae of the genus *Dunaliella* are exceptional in the plant kingdom in their ability to proliferate over practically the entire range of salinities. The algae grow in media with NaCl concentrations ranging from <0.1 M to near saturation, while maintaining a low intracellular ionic concentration (Avron, 1986). Its outstanding salt tolerance makes the genus *Dunaliella* an intriguing model to identify and characterize mechanisms underlying this capacity. Mechanisms of salt tolerance of plants (Hasegawa et al., 2000) and model systems such as yeast (*Saccharomyces cerevisiae*; Serrano, 1996, 1999) are now vigorously pursued. Major aspects addressed include the osmotic regulation by osmolytes, control of water, and ion fluxes and underlying signaling pathways.

*Dunaliella salina*, devoid of a rigid cell wall, overcomes external osmotic variations by adjusting intracellular levels of glycerol to concentrations balancing the external osmotic pressure. Therefore, the cells maintain a constant volume independent of the ex-

ternal salinity (Avron, 1986; Sadka et al., 1991). Mechanisms governing ionic homeostasis and other aspects of salt tolerance of *D. salina* still remain largely unknown.

Identification of genes/proteins of *D. salina* that are preferentially expressed/accumulated under high salinities have previously led us to unravel novel components contributing to salt tolerance. These include two plasma membrane proteins implicated in alleviating salt-imposed limitations on the availability of CO<sub>2</sub> (Fisher et al., 1996) or iron (Fisher et al., 1997, 1998). Furthermore, these extracellularly exposed proteins are unique in remaining active over a very broad range of salinities in contrast to their mesophilic homologs (Fisher et al., 1996). The present study sheds light on still a different aspect of salt tolerance. A cloned cDNA for a salt-inducible gene was found to encode the microsomal enzyme  $\beta$ -ketoacyl-CoA synthase (Kcs) that catalyzes the condensation of acyl-CoAs with malonyl-CoA to yield  $\beta$ -ketoacyl-CoA and CO<sub>2</sub>. This reaction is the first and rate-limiting of four reactions leading to fatty acid elongation by sequential addition of C2 units to acyls of at least C12 (Lessire et al., 1989; Millar and Kunst, 1997). The primary substrates for elongation are the products of de novo fatty acid biosynthesis in the plastid that employs the acyl carrier protein, rather than CoA, as acyl carrier.

Cloning of plant *kcs* genes was initially based on directed transposon tagging in *Arabidopsis* (James et al., 1995) and partial amino acid sequencing of a protein from developing embryos of jojoba (*Simmondsia chinensis*; Lassner et al., 1996). Additional

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<sup>2</sup> Present address: Omrix, Weizmann Science Park, Nes-Ziona 76106, Israel.

<sup>3</sup> Present address: Volcani Center, Bet Dagan 50250, Israel.

<sup>4</sup> Present address: Institute of Biological Research, Nes-Ziona 74100, Israel.

\* Corresponding author; e-mail bczamir@wicc.weizmann.ac.il; fax 972-8-9344118.

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plant genes belonging to this family were identified on the basis of sequence homology or as transposon insertion sites in tagged mutants (Fourman et al., 1998; Millar et al., 1998; Yephremov et al., 1999; Priutt et al., 2000). Functionally characterized plant *kcs* genes include genes expressed in seeds and active in the biosynthesis of storage lipids or waxes (Lassner et al., 1996), as well as genes expressed in vegetative tissues and involved in the biosynthesis of cuticular waxes (Millar et al., 1999; Todd et al., 1999). The *D. salina* Kcs, identified in the context of algal salt responses, is most likely involved in membrane lipid biosynthesis. Specifically, this enzyme may play a role in salt-related changes of microsomal fatty acid composition.

## RESULTS

### Cloning of a Salt-Inducible Kcs from *D. salina*

Cells of *D. salina* transferred from 0.5 to 3.5 M NaCl cease to divide and suffer rapid water loss causing the cells to shrink. Subsequent rapid accumulation of glycerol, to a level osmotically balancing the external salinity, allows the cells to regain their original volume already within approximately 2 h, but cell proliferation resumes only approximately 10 h after the osmotic shock (Sadka et al., 1989; Fisher et al., 1994, 1997). Based on the expectation that genes involved in salt tolerance are preferentially expressed at this time, a cDNA library for poly(A<sup>+</sup>) mRNAs was constructed for *D. salina* cells 9 h after their transfer from 0.5 to 3.5 M NaCl (Fisher et al., 1996). The isolation from this library of a cDNA for a novel salt-inducible gene, cloning of its full length, as well as most of the corresponding genomic sequence are described in "Materials and Methods."

Sequence determination indicated that the cDNA included an open reading frame for a 621-amino acid protein of  $M_r$  69,867 with a predicted pI of 9.26 (Fig. 1; GenBank accession no. AF333040). The nucleotides flanking the assigned initiation codon agree with the core consensus sequence AGNATGNC for translation initiation in plants and animals (Lutcke et al., 1987). Furthermore, an in-frame termination codon is located upstream of the assigned initiation codon (data not shown).

The predicted product of the cloned cDNA belonged to the family of plant  $\beta$ -ketoacyl CoA synthases that act in fatty acid elongation in embryos or vegetative tissues. The sequences included in the alignment (Fig. 1) are of proteins for which Kcs activity has been demonstrated directly or inferred from phenotypes of mutant or transformed plants. The close similarity between the algal and plant proteins extends along most of their length, including the two predicted membrane-spanning domains (Millar et al., 1999), corresponding to residues 111 through 132 and 153 through 175.

However, the predicted algal protein is outstanding in its extended N-terminal sequence: 52 amino acids longer than FDH, the next in N-terminal extension length. N-terminal sequences have not been directly determined yet for any of the mature Kcs proteins. However, results of partial amino acid sequencing of peptides derived from the jojoba Kcs were in keeping with the absence of a cleavable leader peptide of meaningful length (Lassner et al., 1996). Nonetheless, as shown below, the first 46 N-terminal residues are not essential for enzymatic activity. Another difference noted is that only four of the six Cys residues conserved in other Kcs proteins (Todd et al., 1999) are present in the algal Kcs. Recently reported mutagenesis experiments indicated that only a single Cys residue (residue 223 in the Arabidopsis FAE1) was essential for activity (Ghanevati and Jaworski, 2001).

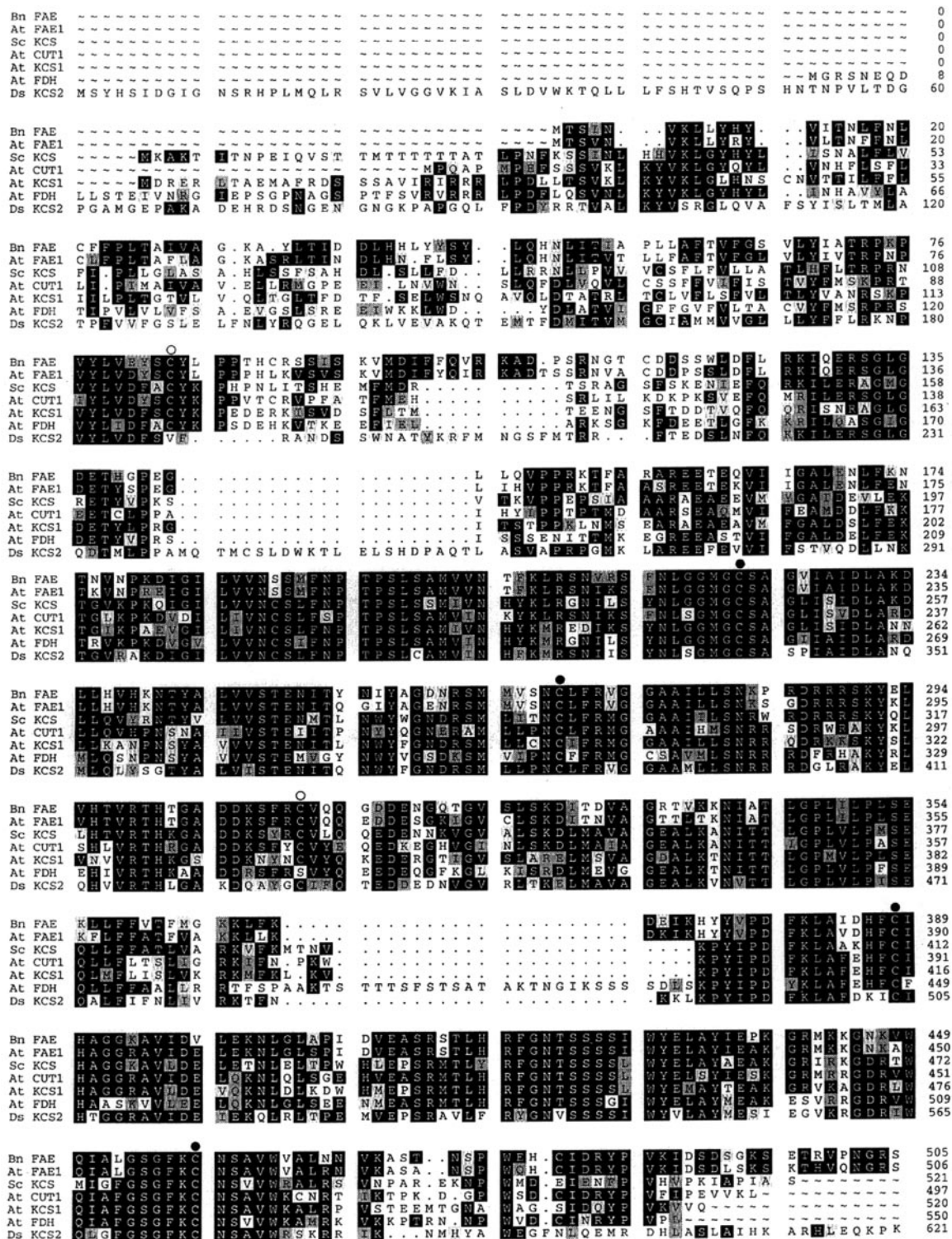
Genomic sequences overlapping most of the cDNA sequence were determined, indicating that the coding sequence is interrupted by at least nine introns (data not shown). Most plant *kcs* genes characterized so far lack introns. Only the FDH gene from Arabidopsis has been shown to include two introns, one of which is positioned close to an intron insertion site (codon 193) in *D. salina* (Yephremov et al., 1999).

### Kcs Activity in *D. salina*

To further study the algal *kcs*, *D. salina* cells were fractionated and assayed to localize Kcs enzymatic activity. A lysate of cells grown in 3.5 M NaCl was first spun to separate chloroplasts, and the supernatant was further resolved into soluble and mixed membrane fractions (fraction 1). Centrifugation of fraction 1 through a glycerol gradient yielded purified plasma membranes (fraction 2). Analyses of fractions 1 and 2 (Table I) for the microsomal marker NADPH cytochrome *c* reductase (Peeler et al., 1989) and for chlorophyll, a thylakoid membrane marker, indicated that fraction 1, but not fraction 2, contained microsomal membranes and that both fractions were largely free of thylakoid membranes. Together with the detection of the *D. salina* plasma membrane proteins Dca (Fisher et al., 1996) and TTf (Fisher et al., 1997) in both fractions 1 and 2 (data not shown), these results led to the conclusion that fraction 1 contained both microsomes and plasma membranes, whereas fraction 2 contained microsome-free plasma membranes. Attempts to obtain a microsomal fraction free of plasma membranes in reasonable yield were unsuccessful.

Analyses for Kcs activity (Table I), using the assay detailed in "Materials and Methods" and characterized in following experiments, revealed significant Kcs activity only in fraction 1, and not in fraction 2. No activity was detected in other cell fractions (data not shown). Thus, Kcs activity was localized to the microsomal fraction, similar to its localization in higher plants.





**Figure 1.** Sequence alignment of Kcs from *D. salina* and plant Kcs/Fae proteins. Protein sequences were aligned by the use of the PILEUP program (Genetics Computer Group, Madison, WI). GenBank accession numbers for the aligned proteins are: Fae from *Brassica napus*, U50771; Fae1 from *Arabidopsis*, U29142; Kcs from *Simmondsia chinensis*, U37088; Cut1 from *Arabidopsis*, AF129511 (recently, *CUT1* was shown to be identical to *CER6*, a gene critical for *Arabidopsis* pollination; Fiebig et al., 2000); Kcs1 from *Arabidopsis*, AF053345; FDH from *Arabidopsis*, ATH010713; and Kcs2 from *D. salina* (denoted elsewhere in this paper as Kcs), AF333040. Black circles, Conserved Cys; white circles, conserved Cys with one exception.

**Table 1.** Characterization of two membrane fractions from *D. salina*

Cell growth and isolation of fractions 1 and 2 were as described in "Materials and Methods." Assay of antimycin A-insensitive NADPH CytC reductase and unit definition were as described (Peeler et al., 1989). Chlorophyll was determined spectroscopically. The characterization and assay of Kcs activity [with 30  $\mu$ g of protein and acyl(18:1)-CoA as the acceptor substrates] are described in "Results" and "Materials and Methods."

	NADPH Cytochrome c Reductase	Chlorophyll	Kcs Activity
	units	$\mu$ g mg protein <sup>-1</sup>	units
Fraction 1	2.03	0.05	37.6
Fraction 2	0.002	nd <sup>a</sup>	3.6

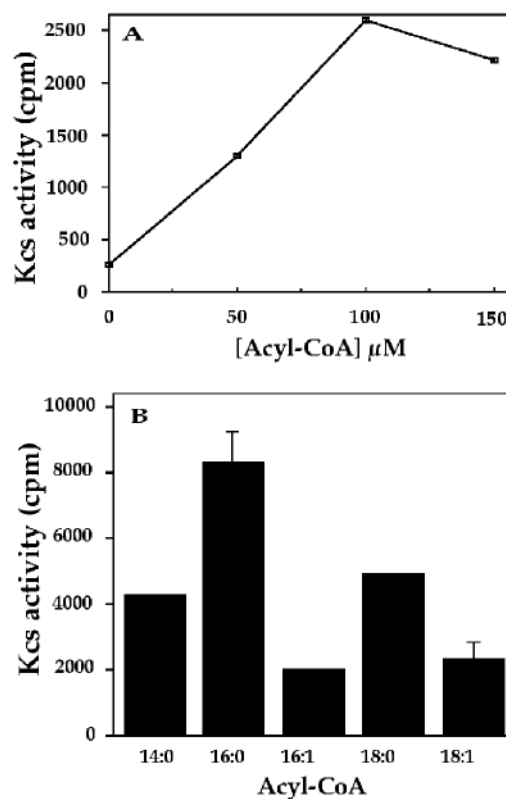
<sup>a</sup> nd, Not detected.

The Kcs activity of fraction 1, first characterized for dependence on enzyme concentration and time course of the reaction (data not shown), was analyzed for its dependence on added acyl-CoA (Fig. 2A). The results clearly demonstrate the dependence of the reaction on acyl(18:1)-CoA, up to 100  $\mu$ M, with only a low background noted in the absence of substrate. Thus, the added acyl-CoA, rather than an endogenous acceptor, served as the predominant substrate for condensation with malonyl-CoA. Assays of the condensing activity with several different acyl-CoAs, at 100  $\mu$ M, were performed to evaluate the substrate preferences of the algal Kcs (Fig. 2B). The results, reflecting relative substrate preferences but not precise affinities, indicated that saturated acyls were preferred (in the order C14:0 < C16:0 > C18:0) over the mono-unsaturated acyls C16:1 and C18:1. As shown below (Fig. 3), the relative efficiencies of the least and maximally effective substrates tested, i.e. acyl(18:1) and acyl(16:0), respectively, were essentially retained over a broad range of enzyme concentrations.

Kcs activity was further characterized for the condensation products of [2-<sup>14</sup>C]malonyl-CoA, with myristyl(14:0)-CoA or palmityl(16:0)-CoA as acceptor substrates (Fig. 4). In this case, the reaction mixtures were supplemented with NADH and NADPH to allow subsequent reduction steps in the formation of the fully reduced acyl moieties. The TLC analysis of the reaction mixtures indicates the formation of condensation products comigrating with palmitic(16:0) or stearic(18:0) acids (as methyl esters), corresponding to the C2-elongated myristyl(14:0)-CoA or palmityl(16:0)-CoA substrates, respectively. The additional, faster migrating spots evident in the chromatogram must correspond to hydroxy or desaturated intermediates in the conversion of the  $\beta$ -ketoacyl primary condensation products into the fully reduced acyls. These results show that the algal Kcs, similar to plant counterparts, catalyzes the condensation of malonyl-CoA with the provided acyl-CoA substrates to form the expected elongated products.

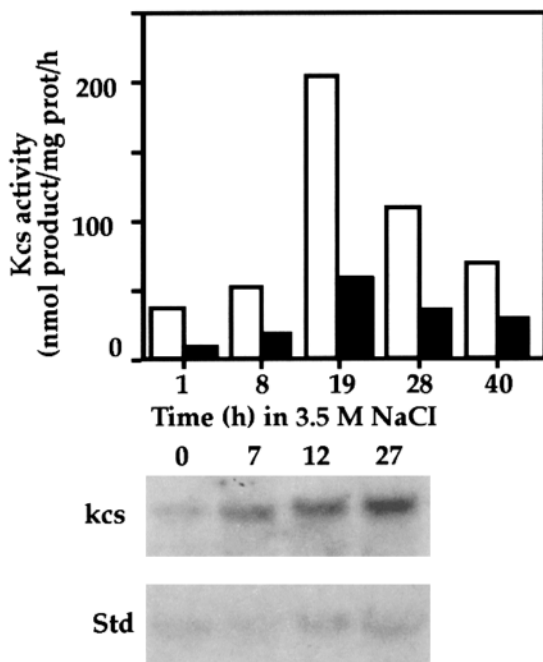
### Salt Induction of *kcs* mRNA Accumulation and Kcs Activity

Levels of *kcs* mRNA and Kcs activity were determined in *D. salina* cells at different times after transfer from 0.5 to 3.5 M NaCl (Fig. 3). In these cultures, cell division started approximately 10 h after transfer. Assays of Kcs activity, with acyl(16:0)-CoA or acyl(18:1)-CoA as acceptor substrates, indicated for both substrates a progressive rise in activity from 8 to 19 h after the salt shock followed by a decline at 28 h. By 40 h and onwards (data not shown) the activity reached nearly a constant level severalfold higher than the activity exhibited by cells grown in 0.5 M NaCl. A similar difference in Kcs activity was observed between cells grown continuously in 0.5 or 3.5 M NaCl. Northern-blot analysis of *kcs* mRNA during the course of adaptation to 3.5 M NaCl indicated an increase in the level of the transcript 7 h after the salt shock and further increases at 12 and 28 h. A reduction in the level of the *kcs* transcript was observed at later times (data not shown). A comparison of the



**Figure 2.** Acyl-CoA dependence of Kcs activity. A, Dependence on Acyl-CoA concentration. Assay mixtures, with 30  $\mu$ g of protein of fraction 1 from cells grown in 3.5 M NaCl, contained the indicated concentrations of acyl(18:1)-CoA. Assay conditions and product analysis were as described in "Materials and Methods." B, Kcs activity with different acyl-CoAs. Activity assays, as described in A, were performed with 100  $\mu$ M of the acyl-CoAs indicated. SD values are indicated for acyl(18:1)-CoA and acyl(16:0)-CoA substrates. Kcs activity, cpm in 0.6 mL of chloroform extracts, as detailed in "Materials and Methods."





**Figure 3.** Analysis of *kcs* transcript and Kcs activity in *D. salina* cells after exposure to high salt. Cultures were transferred, in two steps, from a medium with 0.5 M to a medium with 3.5 M NaCl. Top, Assay of Kcs activity. Cell culture batches of approximately 1 L were removed at the indicated times after transfer to 3.5 M NaCl and the cells were fractionated to give fraction 1. Kcs activity was determined with 30  $\mu$ g of protein of fraction 1 and 100  $\mu$ M acyl(16:0)-CoA (white bars) or acyl(18:1)-CoA (black bars) as described in "Materials and Methods." Bottom, Northern-blot analysis of *kcs* mRNA. Samples of approximately 200 mL were removed at the indicated times after transfer to 3.5 M NaCl and total RNA was extracted. Northern-blot hybridization with *kcs* cDNA (*kcs*) or standard (*std*) probe was as described in "Materials and Methods."

course of change in Kcs activity and transcript accumulation indicates both reach a maximum within 12 to 27 h after the salt shock.

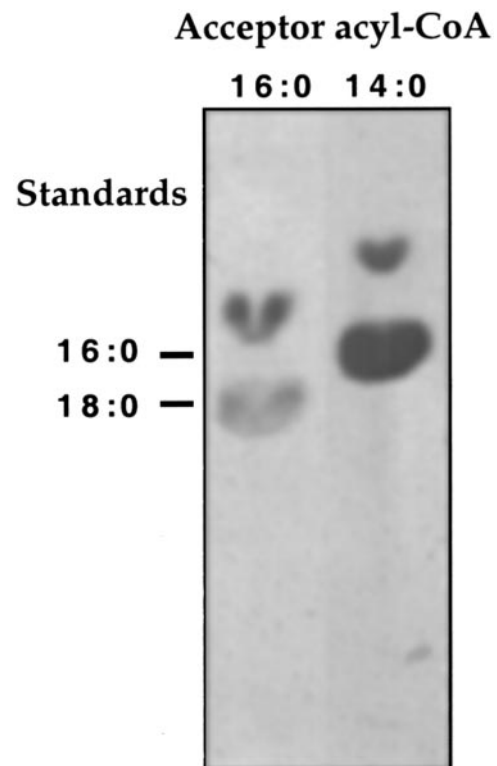
To determine whether *kcs* induction reflected a salt-specific or general osmotic response, the level of the *kcs* transcript was determined in cells subjected to osmotic upshock generated by added glycerol rather than NaCl. The results (data not shown) indicate only a low rise in both *kcs* transcript and Kcs activity, compared with salt-shocked cells. Thus, *kcs* is induced in response to salt to a far greater extent than to nonionic osmotic shock.

#### Functional Expression of *kcs* in *Escherichia coli*

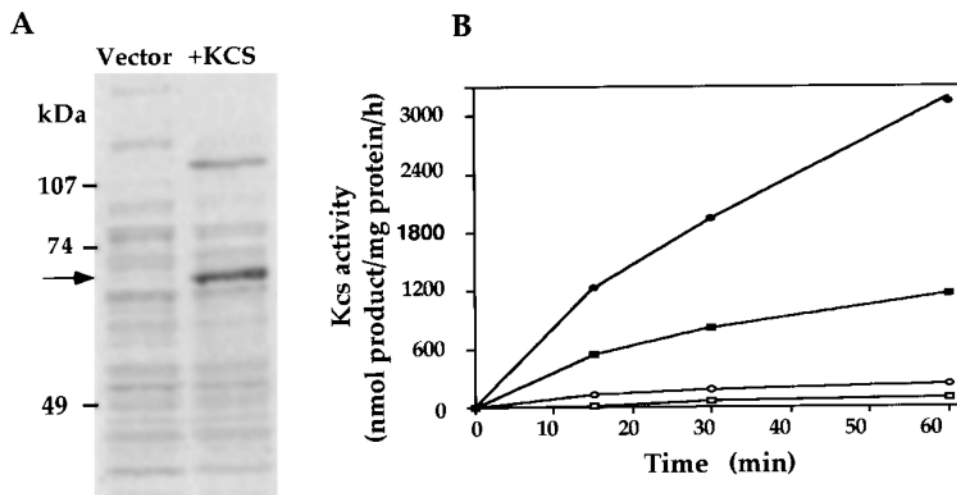
To critically establish that *kcs* encoded Kcs, a cDNA fragment starting at Ser-47 (Fig. 1) was cloned into pET28c, in phase with the initiator ATG and 37 additional N-terminal codons of the vector encoding His tag and T7 tag sequences. Expression of the fusion protein in *E. coli* was monitored by [ $^{35}$ S]Met labeling of isopropyl- $\beta$ -D-thiogalactoside (IPTG)-induced cells treated with rifampicin to enhance ex-

pression selectivity. The results (Fig. 5A) show that cells transformed with the recombinant plasmid, but not with the cloning vector alone, synthesized a protein of the expected size. Another preferentially expressed protein, of >107 kD, likely represents residual Kcs dimers that escaped dissociation by SDS. Evidence for the homodimeric structure of native plant Kcs has been presented (Lassner et al., 1996).

Membrane fractions from *E. coli* transformed with the *kcs* recombinant plasmid, or vector alone, were solubilized and assayed for Kcs enzymatic activity according to a similar protocol as used to assay fraction 1 from *D. salina*. The results (Fig. 5B) clearly show Kcs activity in fractions from transformants with the recombinant plasmid, but not with vector alone, confirming that the cloned gene encoded a functional Kcs enzyme. Furthermore, the results show that the truncation of the N-terminal 46 amino acid residues did not abolish the activity of the expressed protein. The cofractionation of the Kcs activity with a membrane fraction in *E. coli* suggests, but does not prove, the membrane integration of the protein in the heterologous host.



**Figure 4.** Analysis of Kcs reaction products. Kcs assay mixtures with 100  $\mu$ M of the indicated Acyl-CoAs were supplemented with 0.5 mM each NADH and NADPH. Assay conditions, reaction product processing, resolution of the reaction products by thin-layer chromatography (TLC), and autoradiography were as described in "Materials and Methods." Markers: 16:0, methyl ester of [ $^3$ H] palmitic acid; 18:0, methyl ester of [ $^{14}$ C] stearic acid.



**Figure 5.** Expression and activity of Kcs in *E. coli*. A, Detection of heterologously expressed Kcs. The *kcs* cDNA cloned in the pET28c expression vector was transformed into *E. coli*. Transformants with the recombinant plasmid (+) or vector alone (–) were metabolically labeled with [ $^{35}$ S]Met and membrane fractions were analyzed by SDS-PAGE and autoradiography as described in “Materials and Methods.” The major translation product is marked by an arrow. A preferentially labeled >107-kD polypeptide is likely to represent an incompletely dissociated dimeric form of Kcs, as discussed in “Results.” B, *E. coli* transformants with the *kcs* cDNA containing plasmid (black symbols), or the pET28c vector alone (white symbols), were treated under conditions inducing *kcs* expression and a membrane fraction was isolated and assayed for Kcs activity as described in “Materials and Methods.” Reaction mixtures contained 3 (rectangles) or 6 (circles)  $\mu$ g of protein of the *E. coli* fraction.

#### Fatty Acid Composition of Membrane Fractions from Cells Grown in Different Salinities

The salt inducibility of *kcs* in *D. salina* suggested that adaptation to salt might entail the elongation of fatty acids in membrane lipids. To test this possibility, lipids isolated from membrane fractions from low- or high-salt-grown *D. salina* were analyzed for fatty acids composition (Table II). The results are presented as the relative distributions of the major fatty acids in each of the fractions (C14–C22 in fraction 1 and C14–C18 in fraction 2), excluding longer fatty acids present in minute amounts.

The analysis reveals that Fraction 1 from cells grown in 3.5 M NaCl differs from the corresponding fraction from cells grown in 0.5 M NaCl in containing a markedly lower proportion of C16 fatty acids, which is mainly accounted for by an increase in the proportion of C18 fatty acids, and to a lesser degree in the proportion of the minor C14 and C22 fatty acids. On the other hand, the relative proportions of C14, C16, and C18 in fraction 2 remain practically unaltered between cells grown in high and low salt, although an altered distribution of different desaturated C18 fatty acids is noticeable.

In both fractions 1 and 2, the fully saturated 16:0 palmitic acid is the predominant C16 fatty acid, whereas C18 fatty acids mostly consist of desaturated species. Hence, the salt-related shift from C16 to C18 fatty acids in fraction 1 also entails a rise in the overall proportion of desaturated fatty acids. Thus, microsomal membranes of cells grown in high salinity contain a higher proportion of C18 to C16 fatty

acids and, as a consequence, a higher content of desaturated fatty acids, compared with low-salt-grown cells.

#### DISCUSSION

Transcriptional activation of the *D. salina kcs* gene and enhancement of Kcs activity were shown to follow a hyperosmotic salt shock. These responses do not constitute a transient stage in salt adaptation, but persist in cells growing continuously in high salinity.

Plant Kcs enzymes have not been obtained yet in homogeneous, pure form (Lassner et al., 1996; Todd et al., 1999). Studies of mutant or transformed plants or partially purified enzymes led to the conclusion that plant Kcs enzymes vary in their preference for different chain length acyl-CoA substrates, i.e. each enzyme typically participates in several rounds of elongation within a given range of chain lengths (Lassner et al., 1996). The *D. salina* enzyme does not seem to be involved in the formation of very long-chain fatty acids, which are very scarce in the algal membranes, but apparently prefers relatively shorter chain acyl-CoA substrates than enzymes such as the jojoba Kcs (Lassner et al., 1996), or the heterologously expressed Arabidopsis KCS1 (Todd et al., 1999). However, the *D. salina* Kcs resembles these plant enzymes in preferring saturated over mono-unsaturated acyl-CoA substrates.

The salt inducibility of the *D. salina* Kcs raised the possibility that salt adaptation may entail a shift toward longer chain fatty acids in algal lipids. Be-

**Table II.** Fatty acid composition of fractions 1 and 2 from *D. salina* grown in low and high salinities

*D. salina* cells collected from 121 cultures grown to  $\sim 10^6$  cells mL<sup>-1</sup> in the indicated NaCl concentrations were used to isolate fraction 1, containing microsomes and plasma membrane, and fraction 2, containing purified plasma membrane, as detailed in "Materials and Methods" and "Results." Lipid extraction and fatty acid analysis were performed as described in "Materials and Methods." Wt %, wt of the indicated fatty acids relative to their total amount (100%). Fatty acids of longer chain lengths than those indicated were negligible in amount.

Fatty Acid	Fraction 1 (Microsomes/PM <sup>a</sup> )		Fraction 2 (PM)	
	3.5 M NaCl in growth medium	0.5 M NaCl in growth medium	3.5 M NaCl in growth medium	0.5 M NaCl in growth medium
Fatty acid content (wt %)				
14:0	nd <sup>b</sup>	0.39 $\pm$ 0.01	nd	nd
14:1	2.20 $\pm$ 0.1	2.9 $\pm$ 0.17	2.1	0.83 $\pm$ 0.06
14:2	2.56 $\pm$ 2.03	6.63 $\pm$ 0.5	7.8 $\pm$ 0.87	8.3 $\pm$ 0.61
<b>14<sup>c</sup></b>	<b>4.76 <math>\pm</math> 2.11</b>	<b>9.79 <math>\pm</math> 0.44</b>	<b>9.9 <math>\pm</math> 1.04</b>	<b>9.23 <math>\pm</math> 0.64</b>
16:0	75.30 $\pm$ 3.21	48.91 $\pm$ 0.33	41.87 $\pm$ 0.38	39.83 $\pm$ 0.29
16:1	nd	nd	0.87 $\pm$ 0.06	0.63 $\pm$ 0.12
<b>16</b>	<b>75.31 <math>\pm</math> 3.21</b>	<b>48.91 <math>\pm</math> 0.33</b>	<b>42.73 <math>\pm</math> 0.42</b>	<b>40.47 <math>\pm</math> 0.25</b>
18:0	1.39 $\pm$ 0.01	0.46 $\pm$ 0.08	1.43 $\pm$ 0.06	1.5 $\pm$ 0.35
18:1(w6 + w9)	9.57 $\pm$ 2.46	17.45 $\pm$ 1.08	nd <sup>d</sup>	nd <sup>d</sup>
18:2(w6)	1.95 $\pm$ 1.17	8.14 $\pm$ 0.31	8.47 $\pm$ 0.31	16.67 $\pm$ 0.12
18:3(w3 + w6)	1.61 $\pm$ 0.6	2.37 $\pm$ 0.12	31.83 $\pm$ 1.79	28.9 $\pm$ 0.2
18:4(w3)	2.34 $\pm$ 0.74	2.83 $\pm$ 0.31	5.63 $\pm$ 0.55	3.23 $\pm$ 0.06
<b>18</b>	<b>16.40 <math>\pm</math> 4.16</b>	<b>31.25 <math>\pm</math> 1.48</b>	<b>47.37 <math>\pm</math> 0.67</b>	<b>50.3 <math>\pm</math> 0.5</b>
20:0	nd	0.81 $\pm$ 0.27		
20:3	nd	0.58 $\pm$ 0.07		
<b>20</b>	nd	<b>1.39 <math>\pm</math> 0.28</b>		
22:0	nd	1.63 $\pm$ 0.04		
22:1	4.45 $\pm$ 1.2	7.71 $\pm$ 1.14		
<b>22</b>	<b>4.45 <math>\pm</math> 1.2</b>	<b>8.8 <math>\pm</math> 2.08</b>		

<sup>a</sup> PM, plasma membrane. <sup>b</sup> nd, Not detected. <sup>c</sup> Bold, Total wt % fatty acids of the indicated chain length. <sup>d</sup> In some experiments 18:1(w9) was not fully resolved from 18:3(w3).

cause *D. salina* is devoid of waxes and energy storage lipids (Thompson, 1996), we anticipated the shift to occur in membrane lipids. A pronounced salt-induced shift in fatty acid chain length was detected in a fraction containing both microsomes and plasma membranes. Because no shift was observed in purified plasma membranes, we concluded that fatty acid elongation, for which the analysis necessarily provides only a minimal estimate, was confined to the microsomal fraction.

Palmitic acid (C16:0) is the predominant C16 fatty acid in both plasma membranes and microsomes, whereas the bulk of C18 fatty acids is made up of unsaturated fatty acids. Therefore, it is envisaged that palmitic acid is first elongated to stearic acid (C18:0), which subsequently undergoes various degrees of desaturation. By increasing the proportion of C18:0, the elongation reaction thus provides additional substrate for desaturation. Hence, the salt-related modifications concern not only fatty acid chain elongation but also increased overall desaturation.

Modification of membrane lipid fatty acids in plants has been studied mostly in relation to chilling sensitivity. In this instance, increased desaturation of thylakoid fatty acids was causally linked to enhanced salt tolerance (Somerville, 1995). In *D. salina*, increased desaturation was reported in polar lipid fractions from plasma membranes of *D. salina* grown in

high compared with low salinity (Peeler et al., 1989). Another analysis (Al-Hasan et al., 1987) detected a salt-related increase in the relative proportion of linolenic acid (18:3) in the total lipids of another *D. salina* strain.

The establishment of a direct functional link between the cloned *kcs*, fatty acid modifications, and enhancement of salt tolerance must await the development of appropriate genetic tools for *D. salina*. Nonetheless, taken together, our results provide support to the scheme whereby fatty acid elongation, and probably desaturation, contribute to the salt tolerance of *D. salina*. The understanding of this contribution is made difficult by the fact that homeostatic mechanisms maintain the intracellular ionic concentration at a rather low level, regardless of the external salinity (Pick et al., 1986). As a consequence, the endoplasmic reticulum or Golgi apparatus are not exposed to high ionic concentrations. The most pronounced biochemical change in the intracellular milieu is the accumulation of high concentrations of glycerol (in excess of 4.0 M) that osmotically balance the external high salinity. Glycerol is generally thought to be fully compatible with the stability and function of cellular components (Roberts, 2000). Yet, one cannot dismiss the possibility that some cellular components may not operate optimally in the presence of such high levels of glycerol and need to

undergo adaptive modifications exemplified by the present observations.

The function(s)/structures potentially affected by glycerol in *D. salina* remain elusive. Yet, a plausible direction involves the secretory pathway responsible for transport of proteins and lipids between the endoplasmic reticulum and Golgi apparatus by secretory vesicles that bud from one compartment and fuse with another. Vesicle budding and fusion, as well as discrimination of cargo and targeting molecules, depend on specific protein-membrane and membrane-membrane interactions that were shown to be affected by membrane lipid composition (Rothman and Wieland, 1996; Schenkman and Orci, 1996; Weber et al., 1998; Matsuoka and Schenkman, 2000). It is not unlikely that some of these interactions are hindered in high intracellular glycerol concentrations. Hence, the activity of the salt-induced Kcs in *D. salina* may be required, alongside additional activities, to modify membranes of the endoplasmic reticulum and/or Golgi apparatus so as to optimize vesicular transport in cells grown in high salinity. Adaptations of this sort are not likely to be unique to *D. salina* because intracellular accumulation of inorganic or organic solutes is a ubiquitous salt-adaptive, osmoregulatory response in taxonomically varied organisms (Roberts, 2000).

## MATERIALS AND METHODS

### Algae and Growth Conditions

The source of the *Dunaliella salina* strain used in this study, media, and growth conditions were essentially as described (Ben Amotz and Avron, 1983; Sadka et al., 1989; Lers et al., 1990). Axenic cultures were grown continuously in media with 0.5 or 3.5 M NaCl. Cells were osmotically shocked by transferring cultures grown to  $6 \times 10^5$  cells mL<sup>-1</sup> in a medium with 0.5 M NaCl, to a medium with 3.5 M NaCl essentially as previously described (Fisher et al., 1994).

### Isolation and Cloning of *kcs* cDNA

A cDNA library from salt-shocked *D. salina* was constructed as previously described (Fisher et al., 1996). Specifically, *D. salina* cells grown in 0.5 M NaCl were transferred in two steps to 3.5 M NaCl (Fisher et al., 1994). Total RNA was extracted from cells 9 h after transfer to 3.5 M NaCl and cDNA to poly(A<sup>+</sup>) mRNA was synthesized and cloned into the  $\lambda$  Uni-ZAP XR expression vector (Promega, Madison, WI) as described (Fisher et al., 1996). A phage clone initially isolated in a screen with anti-TTf antibodies (Fisher et al., 1997) was subsequently shown to include a cDNA for an unrelated, salt-inducible gene. The approximately 1.0-kb insert in the original clone, including the 3' end of the cDNA, was used to clone the full-length cDNA by the 5'-RACE procedure (CLONTECH Laboratories, Palo Alto, CA). To obtain corresponding genomic DNA sequences, primers based on cDNA sequences were used with templates of genomic DNA, digested by *Sau* 3A, *Hae* III, *Taq* I, or *Msp* I, followed by fragment circularization by ligase, in several consecutive steps of inverted PCR amplification and subsequent cloning.

### Cell Fractionation

*D. salina* cells, grown in 0.5 or 3.5 M NaCl to  $1 \times 10^6$  cells mL<sup>-1</sup>, were collected by centrifugation at 5,000g for 10 min at 4°C. Cell pellets were washed, in accordance with the growth medium, with 0.5 or 3.5 M NaCl in medium buffer (10 mM KCl, 2 mM MgCl<sub>2</sub>, 5 mM  $\beta$ -mercaptoethanol, and 25

mM Tricine-NaOH, pH 7.8), followed by washing with buffers containing 0.9 or 4.6 M glycerol, respectively, in medium buffer. The cell pellets were resuspended in the same buffers to a density of  $3$  to  $5 \times 10^8$  cells mL<sup>-1</sup> and cells were lysed by adding 3 or 4 volumes of medium buffer to cells grown in 0.5 or 3.5 M NaCl, respectively, and gentle stirring of the suspensions for 20 min at 4°C. The lysates were centrifuged at 8,000g for 20 min at 4°C and Na-EDTA (pH 7.5) was added to the supernatants to a final concentration of 5 mM, followed by gentle stirring for 30 min at 4°C and centrifugation at 235,000g for 45 min at 4°C. The supernatants (soluble fraction) were removed and the pellets were resuspended ( $1 \times 10^6$  cell equivalents/100  $\mu$ L) in storage buffer (1 M NaCl, 10% [w/v] glycerol, 5 mM  $\beta$ -mercaptoethanol, 2% [w/v] CHAPS, and 25 mM Tricine-NaOH, pH 7.8) and stored at -80°C until assayed for Kcs activity (Fraction 1). For further fractionation, the pellets were suspended in medium buffer containing 0.2 or 1.2 M glycerol for pellets from cells grown in 0.5 or 3.5 M NaCl, respectively, and the suspensions were centrifuged at 290,000g for 90 min at 4°C. The pellets were resuspended in 0.3 mL of the respective suspension buffers and loaded on top of glycerol step gradients containing 5 mL of 30% (v/v) glycerol in 30 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM  $\beta$ -mercaptoethanol, and 25 mM mM Tricine-NaOH (pH 7.8) layered on top of 6 mL of 60% (v/v) glycerol in the same buffer. The gradients were spun in swinging buckets at 210,000g for 2 h at 4°C. Pellets (from  $1 \times 10^6$  cell equivalents) were resuspended in 50  $\mu$ L of medium buffer and the suspensions were spun at 5,000g for 10 min at 4°C. The supernatants (fraction 2) were stored in liquid N<sub>2</sub> until analyzed.

### Fractions for Lipid Analysis

The procedure was essentially as described above, but with slight modifications. The medium buffer contained 10 mM Tris-MOPS (pH 7.5) instead of Tricine-NaOH, and contained no  $\beta$ -mercaptoethanol. In all steps after cell lysis, the buffers contained 1 mM benzamide and 5 mM  $\epsilon$ -aminocaproic acid. Fraction 1 pellets were suspended in 3.0 mL and fraction 2 pellets were suspended in 1.5 mL of medium buffer, containing 0.2 or 1.2 M glycerol, for pellets originating in cells grown in 0.5 or 3.5 M NaCl, respectively.

### Kcs Assay

The assay of Kcs activity was essentially similar to previously described protocols (Fehling et al., 1992; Lassner et al., 1996). Specifically, 30  $\mu$ L of reaction mixtures contained 25 mM Tricine-NaOH (pH 7.8), 0.38% (w/v) CHAPS, 0.3 M NaCl, 2 mM  $\beta$ -mercaptoethanol, 17  $\mu$ M [2-<sup>14</sup>C]malonyl-CoA (specific activity: 56 mCi mmol<sup>-1</sup>, Amersham, Buckinghamshire, UK), 100  $\mu$ M of the specified acyl-CoAs (Sigma, St. Louis), and fractions from *D. salina* or transformed *Escherichia coli* (as specified). The mixtures were incubated on a gyratory shaker for 1 h at 30°C. The reaction was stopped by adding 30  $\mu$ L of 20% (w/v) KOH in water:methanol (9:1, v/v) and incubating at 80°C for 1 h. The mixtures were acidified with 30  $\mu$ L of 5 M H<sub>2</sub>SO<sub>4</sub> and extracted twice with 0.6 mL of chloroform. The pooled chloroform extracts were washed twice with 1 mL of water and radioactivity in 0.6-mL aliquots of the chloroform solution was determined in a liquid scintillation counter. Enzyme activity is presented as the cpm measured in such aliquots, or by units defined by nmol of condensation product/mg protein/h.

### Reaction Product Analysis

The reaction conditions, with 30  $\mu$ g of protein of *D. salina* fraction 1 as the source of enzyme, were essentially as described above but the mixtures were supplemented with 0.5 mM each of NADH and NADPH to allow the elongation to proceed beyond the condensation step. The mixtures were processed as in the assay described above and 0.6-mL aliquots of the chloroform extracts were incubated in sealed tubes with 0.5 mL of 10% (w/v) BCl<sub>3</sub> in methanol for 30 min at 70°C. After addition of 2.5 mL of water, the fatty acid methyl esters were extracted twice with 2 mL of hexane. The combined extracts were concentrated under N<sub>2</sub> and resolved on KC18 reverse-phase TLC plates (Whatman, Clifton, NJ) developed in acetonitrile:tetrahydrofuran (80:20, w/v) essentially as described (Evenson and Post-Beittenmiller, 1995), and exposed to BioMax MS film with BioMax transcreen (Eastman-Kodak, Rochester, NY) for 4 d at -80°C.



## Heterologous Expression of *kcs* in *E. coli*

A *kcs* cDNA fragment, extending from the T residue at position 139 (start of the codon for Ser-47, compare with Figs. 1 and 2) to the poly(A<sup>+</sup>) tail, was isolated from the plasmid rescued from the original  $\lambda$ Zap recombinant phage (Fisher et al., 1996) by restriction with *Eco*RI and *Xho*I. The cDNA fragment was ligated into the pET28c vector (Novagen, Madison, WI), previously digested with *Eco*RI and *Xho*I, and the recombinant plasmid was transformed into and propagated in *E. coli* HB101. Shortly before analysis, the recombinant plasmid, or the pET28c vector, was transformed into *E. coli* BL21(DE3) pLysS and fresh transformant clones were grown at 26°C to an optical density at 600 nm (OD<sub>600</sub>) of 0.6 in 20 mL of 2 $\times$  Luria-Bertani medium containing 30  $\mu$ g mL<sup>-1</sup> kanamycin and 34  $\mu$ g mL<sup>-1</sup> chloramphenicol. Kcs synthesis was induced by the addition of 1 mM IPTG and incubation for 3 h at 26°C. The cultures were cooled on ice, centrifuged at 5,000g for 5 min at 4°C, and the cell pellets were washed with 20 mL of sonication buffer (1 M NaCl, 5 mM  $\beta$ -mercaptoethanol, and 25 mM Tricine-NaOH, pH 7.8) and resuspended in 0.5 mL of the same buffer containing 1 mM phenylmethyl sulfonyl fluoride. The cells were lysed by three 20-s pulses of sonication (Ultrasonic Cell Disruptor, model XL2005, Microson, Misonix, NY), the sonicates were cleared by 1 min of centrifugation at 5,000g at 4°C, and the supernatants were spun at 235,000g for 45 min at 4°C. The pellets were resuspended in sonication buffer containing 10% (w/v) glycerol and 2% (w/v) CHAPS and stored at -80°C until assayed for Kcs activity as the *D. salina* fractions. To detect the recombinant Kcs, *E. coli* transformants grown to 0.6 OD<sub>600</sub> in 10 mL of Luria-Bertani medium with the antibiotics indicated above were collected by centrifugation at 5,000g for 15 min at room temperature and the pellets were washed twice with 1 mL of M9 medium and resuspended in 1 mL of M9 medium containing Glc and an amino acid mixture without Met. After 1 h of incubation at 37°C, 1 mM IPTG was added and incubation was continued for 30 min at 37°C followed by the addition of 0.4 mM rifampicin (Sigma). After 20 min at 37°C, 20  $\mu$ Ci [<sup>35</sup>S]Met (specific activity: 1,000 Ci mmol<sup>-1</sup>, Amersham) was added and the cells were incubated for an additional 30 min at 37°C. Nonradioactive Met was added to 40  $\mu$ g mL<sup>-1</sup> and the cultures were kept in ice for 10 min and centrifuged at 5,000g for 10 min at 4°C. Cell pellets were washed with 10% (w/v) Suc and 10 mM Tris-HCl (pH 8.0), resuspended in 80  $\mu$ L of 1 mM Na EDTA, 25 mM KCl, 1 mM dithiothreitol, and 50 mM Tris-HCl (pH 8.0), and the suspensions were subjected to two cycles of freezing in liquid N<sub>2</sub> and thawing at room temperature. After adding 10  $\mu$ L each of 20  $\mu$ g mL<sup>-1</sup> DNase I and 10 mM MgCl<sub>2</sub>, the suspensions were again frozen, thawed, and then clarified by 10 min of centrifugation at top speed in a microfuge at 4°C. The supernatants, 40  $\mu$ L per lane, were resolved on 10% (w/v) SDS-PAGE followed by autoradiography.

## Fatty Acid Analysis

Lipids were extracted as described (Bligh and Dyer, 1959) from 0.9-mL aliquots (1  $\times$  10<sup>10</sup> cell equivalents) of fraction 1 or 2. The chloroform extracts were concentrated under N<sub>2</sub> to 0.1 to 0.2 mL, and mixed with 1 mL of 1.2 N NaOH in 50% (w/v) aqueous methanol. The tubes, tightly sealed with Teflon caps, were placed in a boiling water bath for 30 min, cooled to room temperature, and the solutions were brought to pH  $\leq$  2 with 0.5 mL of 6 N HCl. To form fatty acid methyl esters, 1 mL of 12% (w/v) FCl<sub>3</sub> in methanol was added, the tubes were tightly sealed with Teflon caps, mixed gently, and heated in a water bath at 85°C for 20 min. After cooling to room temperature, 1 mL of hexane:diethyl ether (1:1, w/v) was added and after mixing and phase separation, the bottom phase was discarded and 3 mL of 0.3 N NaOH was added to the upper phase. After mixing and phase separation, the top phase was removed and stored at -80°C until analyzed by gas chromatography, essentially as described by Landry (1994).

## Total RNA Isolation and Northern-Blot Hybridization

Total RNA, prepared from samples harvested at different times after osmotic shock, was analyzed (30  $\mu$ g per lane) by northern blot-hybridization essentially as previously described (Fisher et al., 1994). The hybridization probe used was a 1.2-kb 5' end fragment of the *kcs* cDNA. The probe used as a hybridization standard was as described (Fisher et al., 1997).

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