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Genetic and Metabolic Controls for Sulfate Metabolism in *Neurospora crassa*: Isolation and Study of Chromate-Resistant and Sulfate Transport-Negative Mutants

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Mutants of *Neurospora* resistant to chromate were selected and all were found to map at a single genetic locus designated as *cys-13*. The chromate-resistant mutants grow at a wild-type rate on minimal media but are partially deficient in the transport of inorganic sulfate, especially during the conidial stage. An unlinked mutant, *cys-14*, is sensitive to chromate but transports sulfate during the mycelial stage at only 25% of the wild-type rate; *cys-14* also grows at a fully wild-type rate on minimal media. The double-mutant strain, *cys-13;cys-14*, cannot utilize inorganic sulfate for growth and completely lacks the capacity to transport this anion. The only biochemical lesion that has been detected for the double-mutant strain is its loss in capacity for sulfate transport. *Neurospora* appears to possess two distinct sulfate permease species encoded by separate genetic loci. The transport system (permease I) encoded by *cys-13* predominates in the conidial stage and is replaced by sulfate permease II, encoded by the *cys-14* locus, during outgrowth into the mycelial phase. The relationship of these new mutants to *cys-3*, a regulatory gene that appears to control their expression, is discussed.

Genetic and metabolic controls over enzyme synthesis in the pathway for sulfate assimilation in *Neurospora* appear to involve both positive and negative elements (9). Methionine, or a metabolic derivative of it, acts in a negative fashion as a corepressor for severe repression of the synthesis of a family of related enzymes including aryl sulfatase, choline sulfatase, choline-O-sulfate permease, and sulfate permease (10). Positive control is also indicated for this system, because a functional product of the *cys-3* locus appears to be required for the synthesis of these same enzymes, *cys-3* mutants having a pleiotropic loss of all of these activities (9).

The first step for sulfate assimilation may be considered to be the entrance of the ion to form an intracellular pool of inorganic sulfate. Sulfate transport in *Neurospora* is a specific, energy-dependent process and seems to be a major point of control for the entire pathway since an end product, methionine, strongly represses sulfate transport. In addition, mutants of the *cys-3* regulatory gene cannot utilize sulfate for growth and completely lack the capacity for sulfate transport, presumably because *cys-3*$^+$ is required to activate the structural gene that encodes the sulfate permease. To further these studies, it seemed desirable to isolate and study mutants that specifically affect the process of sulfate transport and to determine whether such genetic loci are tightly linked to the *cys-3* gene in an operon-like arrangement to facilitate genetic control.

To isolate sulfate transport-negative mutants, strains resistant to the toxic analogue, chromate, were selected. Although all of the chromate-resistant mutants showed a decreased capacity for sulfate transport, they, nevertheless, all grew at the wild-type rate on minimal medium, even when growth-limiting concentrations of sulfate were employed. This paper reports findings with these chromate-resistant strains and presents clear evidence to show that *Neurospora* actually possesses two distinct sulfate transport systems. The presence of the different sulfate permease species in certain developmental stages will be discussed.

MATERIALS AND METHODS

Neurospora strains. The wild-type strain 74-OR23-1A of the Oak Ridge genetic background and various

cysteine mutants originated from the Fungal Genetics Stock Center, Dartmouth College, Hanover, N. H. The *cys-11* (NM86) mutant strain and *cys-5* (85518) are apparently allelic since both lack adenosine triphosphate (ATP)-sulfurylase activity. Crosses were carried out on the medium of Westergaard and Mitchell (16). Standard techniques were utilized for spore activation and the analysis of random ascospores.

Selection of mutants. A suspension of wild-type conidia was adjusted to 10^5 spores per ml and irradiated with an ultraviolet lamp to yield 1 to 5% survival; 1 ml of the irradiated suspension was spread on plates containing Fries medium (2) lacking the normal sulfur supply but supplemented with 0.25 mM methionine, 25 mM potassium chromate, and a mixture of sucrose and sorbose to induce colonial morphology. The chromate was sterilized by filtration. After several days of growth, colonies which appeared were selected and transferred to slants. Chromate resistance was tested by inoculating a conidial suspension into liquid medium containing 0.25 mM methionine and 5 mM chromate and observing the tubes for growth at 4 to 5 days. Wild type failed to grow at all in this test medium, whereas chromate-resistant mutants grew rapidly under these conditions.

Growth studies. Flasks containing 40 ml of Fries liquid minimal medium with the sole sulfur supply as 0.02, 0.1, or 2 mM inorganic sulfate or 10 mM methionine were prepared. Such flasks were inoculated in triplicate with conidial suspensions of wild type, *cys-13* (w-4), *cys-14* (p2), or the double mutant, *cys-13;cys-14*. After standing growth for 2, 3, and 4 days, one mycelia pad for each strain was collected, washed, dried, and weighed. In a similar fashion, growth tubes containing Fries minimal medium which was solidified with 1.5% agar and contained various concentrations of sulfate were inoculated with the same strains, and the linear growth rate at 25 C was determined.

Sulfate transport studies. The rate of sulfate transport was determined by incubating conidia for 15 min or mycelia for 4 min at 25 C in Fries minimal medium containing 1 mM radioactive sulfate. At the appropriate times, the cells were collected on glass fiber filters, dried, and counted for radioactivity in a gas flow, thin-window planchet counter (Nuclear-Chicago Corp., Des Plaines, Ill.). More detailed methods for assay of the sulfate permease of *Neurospora* will be described elsewhere.

Chemicals. Radioactive sulfate ($H_2S^{35}O_4$) was purchased from New England Nuclear Corp., Boston, Mass. Choline-O-sulfate was chemically synthesized (3) and repeatedly crystallized from ethyl alcohol until completely free of inorganic sulfate. All other chemicals were the purest available from commercial sources.

RESULTS

Isolation of chromate-resistant mutants. Chromate-resistant mutants were selected from plates containing minimal medium supplemented with methionine and chromate. The mutant strains were purified by crossing with wild type and obtaining chromate-resistant isolates. It was predicted that at least a predominate group of chromate-resistant strains would show a concomitant loss in the ability to utilize inorganic sulfate; however, in every case, the resistant strains, designated as *cys-13*, grew on minimal medium (Table 1). Different cysteine mutants that have genetic blocks at various steps in the sulfate assimilatory pathway were also tested for their resistance to chromate (Table 1). All other cysteine mutants tested are clearly sensitive to the analogue, except for *cys-3*, the regulatory mutant that lacks all sulfate transport ability. It is particularly instructive to note that *cys-11* (NM86) and *cys-5* (85518) are chromate-sensitive, since these two mutant strains lack ATP-sulfurylase activity, the first enzymatic step in the assimilation of sulfate. These results suggest that to acquire resistance to chromate, it is necessary to prevent entry of the inhibitor, requiring a mutant that affects a permease function. Only *cys-3* and *cys-13* mutants display chromate resistance, and, as will be shown later, both do affect the transport of inorganic sulfate.

In one instance, a colony was isolated which had grown on the chromate selection medium; however, when retested, this strain was clearly sensitive to the analogue. This particular strain proved to have a distinct new mutation, designated as *cys-14*, which also affects sulfate transport; *Cys-14* also grows upon minimal medium (Table 1).

Genetic homogeneity of chromate-resistant mutants. Many of the chromate-resistant strains were individually crossed with wild type and invariably yielded a 1:1 ratio of sensitive to resistant isolates, showing that single genetic changes were responsible for the acquisition of chromate resistance (Table 2). Representative chromate-resistant mutants were intercrossed to see whether mutation at distinct genetic loci conferred resistance. Table 2 shows that no sensitive segregants at all were obtained from such crosses, indicating that the various resistant strains isolated in this study are alleles of the same gene, designated here as *cys-13*. To localize the locus responsible for resistance to chromate, *cys-13* was crossed with the multiply marked *alcoy* strain, but showed no clear linkage to any of the genetic markers carried by *alcoy;* however, it was noted that resistance was linked to mating type (Table 3). Crosses of *cys-13* with *leu-3*, a gene that marks the left arm of chromosome 1, resulted in about 25% recombination, whereas chromate resistance was found to be very tightly linked to the *his-3* locus, located on the right arm of chromosome 1. With the use of mating type as an outside marker, the location of *cys-13* was de-

TABLE 1. *Growth of Neurospora strains on various sulfur sources*[a]

Locus	Allele	Sulfur source for growth		
		Sulfate	Choline-O-sulfate	Methionine + chromate
Wild type		+	+	−
cys-1	84605	−	−	−
cys-2	80702	−	−	−
cys-3	P22	−	−	+
cys-4	K7	−	−	−
cys-5	85518	−	−	−
cys-5	NM44	−	−	−
cys-9	T156	−	−	−
cys-11	NM86	−	−	−
cys-12	NM286t	−	−	−
cys-13	P1	+	+	+
cys-13	W2	+	+	+
cys-13	W4	+	+	+
cys-13	W16	+	+	+
cys-13	W17	+	+	+
cys-13	W18	+	+	+
cys-13	W27	+	+	+
cys-13	WB7	+	+	+
cys-13	WB13	+	+	+
cys-13	WB27	+	+	+
cys-13	WB31	+	+	+
cys-13	WE10	+	+	+
cys-13	SE46	+	+	+
cys-14	P2	+	+	−
cys-13;cys-14	P1, P2	−	+	+

[a] A light conidial inoculum of each strain was placed into Fries minimal liquid medium with the sole sulfur source provided as 2 mM sulfate, 5 mM choline-O-sulfate, or 0.25 mM methionine plus 5 mM potassium chromate. Growth was recorded as positive (+) or negative (−) after 4 days of incubation at 25 C, except for *cys-12* which was incubated at 37 C. All strains tested grow well on medium supplemented with methionine.

termined to be approximately 2 map units to the right of *his-3*. As expected, independent crosses showed that several independently isolated *cys-13* alleles all displayed tight linkage to *his-13* (Table 3).

Crosses of cys-13 with cys-14. As mentioned above, both *cys-13* and *cys-14* strains grow on minimal medium; however, when *cys-13* (resistant) was crossed with *cys-14* (sensitive), auxotrophic recombinants were obtained which are chromate-resistant and unable to utilize inorganic sulfate (Table 4). The results show that such auxotrophic recombinant isolates were obtained when *cys-14* was crossed with five different alleles of the *cys-13* gene, and their frequency of appearance (25%) indicates that the *cys-13* and *cys-14* loci are unlinked. As predicted, the crosses give

TABLE 2. *Intercrosses of chromate-resistant strains by each other and by wild type*[a]

Cross	No. of isolates	
	Sensitive	Resistant
W4 × WB13	0	32
W4 × W17	0	43
W4 × WB31	0	62
W4 × BH2	0	59
W4 × W16	0	33
WB31 × BH2	0	43
WB31 × WB13	0	30
WB31 × wild type	16	28
W16 × wild type	15	17

[a] Random ascospores from crosses of chromate-resistant strains with each other and with wild type were isolated; a light conidial inoculum for each isolate was tested for chromate resistance in liquid Fries medium containing 0.25 mM methionine and 5 mM potassium chromate.

TABLE 3. *Crosses of cys-13 alleles by other genetic markers*[a]

Cross	Genetic marker	Total no. of isolates	No. of recombinants	Recombination
				%
cys-13 × *al-1*	*al-1*	55	22	40
cys-13 × *leu-3*	*leu-3*	32	8	25
cys-13 × wild type	Mating type	148	30	20
cys-13 (W4) × *his-3*	*his-3*	23	0	
cys-13 (WB31) × *his-3*	*his-3*	57	2	
cys-13 (W16) × *his-3*	*his-3*	30	1	
cys-13; cys-14 × *his-3*	*his-3*	122	3	
his-3, cys-13; cys-14 × wild type	*his-3*	78	0	1.9

[a] Individual isolates derived from random ascospores were tested for the various genetic markers utilized. The value for recombination of *cys-13* with *his-3* is derived by combining all of the data for crosses with *his-3*.

rise to prototrophic sensitive and resistant progeny as well as the new class of resistant, sulfate nonutilizing isolates. This outcome suggests that *Neurospora* possesses two sulfate transport systems and that mutational loss of both must occur before an inability to utilize inorganic sulfate for growth is realized.

Growth rate of cys-13 and cys-14 strains. Preliminary assays confirmed the prediction that these mutants had a diminished capacity for sulfate transport. It was thus of interest to determine whether the growth rate was decreased when the only source of sulfur was presented as inorganic sulfate. The results shown in Table 5 clearly demonstrate that both the *cys-13* and the

cys-14 strains grow at a wild-type rate even when the sulfate concentration was decreased so as to severely limit the rate of growth. The double-mutant strain, *cys-13;cys-14*, shows no growth under these conditions unless the medium is supplemented with methionine, when it grows at wild-type rate. These strains were also compared for linear growth rate in growth tubes and again both *cys-13* and *cys-14* grew at the wild-type rate; even the double mutant strain grew to some extent under these conditions, presumably by utilizing sulfur-containing impurities present in the agar.

Localization of cys-14. Since *cys-14* alone has no phenotypic effect that we can detect, the mapping of this gene had to be somewhat indirect. The procedure utilized was to cross *his-3,cys-13;-cys-14* with *alcoy*, the multiply marked strain, and with *cot-1,inos*. Since *his-3* and *cys-13* are tightly linked, *his-3* ascospores were selected and invariably proved to be *his-3,cys-13* as proved by resistance to chromate. The *his-3,cys-13* isolates were then tested for the presence of *cys-14* since a mutant allele at this locus results in a methionine requirement, whereas *his-3,cys-13;cys-14*$^+$ only requires histidine for growth. By following the recombination of other genetic markers with the methionine requirement, it was obvious from the the cross with *alcoy* that *cys-14* was carried on chromosome 4 or 5 since it showed linkage to *cot-1*. A similar cross with *cot-1,inos* demonstrated that *cys-14* is located on chromosome 4 and resides approximately 21 map units from *cot-1;* a more exact localization of *cys-14* has not yet been carried out.

Transport of inorganic sulfate. The transport of the sulfate ion in *Neurospora* requires metabolic energy, is highly temperature-dependent, and is regulated by repression with methionine (*unpublished data*). It was predicted that chromate resistance would result from a mutational loss of sulfate permease. Thus, it was of considerable interest to examine the uptake of inorganic sulfate in wild type, *cys-13*, *cys-14*, and in the *cys-13;cys-14* double-mutant strain. The transport studies clearly show that both the *cys-13* and *cys-14* mutants have partial, stage-specific deficiencies in sulfate uptake, and that the double mutant completely fails to transport this ion (Table 6). The *cys-13* mutant shows negligible sulfate transport in the conidial stage but mycelia of this strain transport the ion at a wild-type rate. Conversely, the *cys-14* mutant has a normal complement of the conidial permease but only about 25% of the wild-type level in the mycelial phase. These results suggest that *cys-13* and *cys-14* loci each encode a distinct form of sulfate permease, one of which is nearly exclusively present in conidia, whereas the other predominates in the mycelia stage. The double mutant, obtained by recombination of *cys-13* and *cys-14*, lacks both permease species and thus is incapable of internalizing sulfate. It is noteworthy that the single mutant, *cys-3*, also completely lacks sulfate transport. The results of crosses of *cys-13;cys-14* with *cys-3* conclusively proved the distinctness of *cys-3* from either *cys-13* or *cys-14*, since *cys-3* is unlinked to either of these mutants.

Growth tests on alternative sulfur sources. To further substantiate the evidence that either single

TABLE 4. *Recombination of cys-14 with various cys-13 alleles*[a]

Cross	Sulfate nonutilizers	Total no. of isolates
cys-13 (P1) × *cys-14*	7	14
cys-13 (WB31) × *cys-14*	9	24
cys-13 (WB13) × *cys-14*	5	25
cys-13 (W16) × *cys-14*	3	30
cys-13 (W4) × *cys-14*	6	27

[a] Individual isolates from the crosses were grown on medium supplemented with methionine and then tested for growth on minimal media. When the data for the five crosses are combined, the sulfate nonutilizing isolates comprise 25% of the total progeny.

TABLE 5. *Growth rate of wild-type and mutant strains in liquid medium*[a]

Strain	Time of growth (days)	Growth (mg, dry weight) with			
		Sulfate			Methionine (10 mM)
		0.02 mM	0.1 mM	2.0 mM	
Wild type	2	8.2	24	31	
	3	10	36	62	68
	4	13	37	79	
cys-13 (W4)	2	9	27	52	
	3	13	40	75	71
	4	14	45	120	
cys-14 (P2)	2	9	28	49	
	3	12	37	73	69
	4	15	44	88	
cys-13 (P1);*cys-14* (P2)	2	0	0	0	
	3	0	0	0	69

[a] Flasks containing liquid medium with the sole sulfur source as indicated were inoculated with equal amounts of conidia of the various strains. The amount of growth that occurred was determined by measuring the dry weight.

mutant or the *cys-13;cys-14* double mutant affects only sulfate transport, their growth response on a variety of sulfur-containing compounds was tested (Table 7). The use of choline-O-sulfate by *Neurospora* involves the transport of the intact molecule, followed by intracellular hydrolysis to yield inorganic sulfate; thus, this compound provides an alternative route for acquisition of an internal pool of sulfate that is independent of a functional sulfate permease. It is particularly instructive to note that *cys-11*, which lacks ATP-sulfurylase, the first enzyme of sulfate assimilation, cannot utilize choline-O-sulfate for growth, whereas the *cys-13;cys-14* strain grows well on this sulfate ester. This result indicates that the pathway of sulfate metabolism is intact in the double mutant and that only sulfate transport capacity is lacking in this strain. The single mutant, *cys-3*, which lacks both the choline-O-sulfate permease and choline sulfatase, cannot utilize choline-O-sulfate for growth.

The double mutant, *cys-13;cys-14*, was found capable of utilizing for growth every sulfur-containing compound tested except for inorganic sulfate and thiosulfate, which share the common uptake system. On the other hand, *cys-3* fails to grow on any of these compounds except for sulfite, cysteine, and methionine (Table 7).

TABLE 6. *Transport of sulfate by conidia and mycelia of wild-type and mutant strains*[a]

Strain	Wild-type rate of transport	
	Conidia	Mycelia
	%	%
Wild type	100	100
cys-3	0	0
cys-13 (permease II)	0–5	100
cys-14 (permease I)	100	25
cys-13;cys-14	0	0

[a] Conidial uptake was measured for 10 min at 25 C by using 1-ml portions with a final conidial suspension to yield an optical density at 420 nm of 0.5. Mycelial transport was assayed for 4 min at 25 C. The uptake by the transport mutants is compared with the wild-type strain which is given an arbitrary value of 100% for both mycelial and conidial sulfate transport. The values obtained for wild type are 1,135 counts per min for conidia and 13,072 counts per min per mg of protein for mycelia.

TABLE 7. *Utilization of various sulfur-containing compounds for growth by wild type, cys-3, and cys-13;cys-14*[a]

Compound	Growth response of		
	Wild type	*cys-13; cys-14*	*cys-3*
Sulfate	+	−	−
Sulfite	+	+	+
Thiosulfate	+	−	−
Cysteine	+	+	+
Cysteic acid	+	+	−
S-methylcysteine	+	+	−
S-ethylcysteine	+	+	−
Taurine	+	+	−
Homocysteic acid	+	+	−
Choline-O-sulfate	+	+	−
α-Methyl methionine	+	+	−
Methionine	+	+	+

[a] A light conidial inoculum of each strain was placed into Fries medium containing the designated compounds at a final concentration of 2 mM as the sole sulfur source. Growth was clearly positive (+) or negative (−) after 4 days of incubation at 25 C.

DISCUSSION

The results presented here demonstrate that *Neurospora* possesses two distinct sulfate transport systems. These two permease species appear to be encoded by structural loci unlinked to each other or to the regulatory *cys-3* gene. The *cys-13* locus, which apparently encodes sulfate permease I, is carried on chromosome 1; this permease form predominates in the conidial stages and also appears to be responsible for the lethal entry of chromate since *cys-13* mutants are uniformly very resistant to this analogue. Sulfate permease II, encoded by *cys-14*, is the form mainly present in the mycelial phase. More detailed biochemical studies of sulfate transport indicate that the conidial permease can be distinguished from permease II in that the former has a dramatically higher K_m for inorganic sulfate. It also appears that the sulfate transport of *cys-14* mutants during the mycelial phase is not typical but is due to the continued presence of the conidial permease species. Thus, the two distinct permease systems for *Neurospora* each seem to be present at particular stages of the life cycle such that the conidial sulfate permease I is replaced or greatly supplemented during outgrowth into the mycelial phase by permease II. This apparent stage specificity suggests that additional controls, beyond those that regulate both permease forms, determine the particular developmental stage at which the individual structural genes function.

It appears that at least the primary function of both permease forms is for sulfate uptake, since *cys-3* mutants lack both forms and since both are repressed by methionine. The only biochemical

lesion that has been detected for *cys-13,cys-14*, or the *cys-13;cys-14* double mutant is in sulfate transport capacity; clearly the complete assimilatory pathway for sulfate is intact in these strains. It is also noteworthy that the *cys-13;cys-14* strain can utilize a wide variety of sulfur compounds for growth, whereas the *cys-3* mutant fails to respond to nearly all external sulfur sources. The *cys-3* mutant apparently either fails to recognize or cannot respond to a sulfur limitation with the synthesis of a whole family of enzymes related to sulfur procurement. It appears that *cys-3* has a relatively intact pathway for sulfate assimilation, but that all leads into the sequence from various environmental sulfur sources that require the synthesis of specialized enzymes are lacking. The *cys-3* gene clearly exerts control over both the *cys-13* and *cys-14* loci since *cys-3* mutants lack all sulfate transport ability. Since *cys-3* is unlinked to the genetic loci that it appears to regulate, the control may be mediated by a diffusible, regulatory compound, presumably a macromolecule (8).

Of the various microorganisms which have been studied, only *Neurospora* appears to have two, distinct transport systems for sulfate. Arst (1) found that *s-3* mutants of *Aspergillus* were deficient in sulfate transport and were uniformly more leaky than mutants of other steps in the pathway. Possibly the slow growth of *s-3* mutants is afforded by a separate, minor permease that is not developed to the extent found in *Neurospora* where either permease individually supports normal growth. Chromate-resistant mutants of *Salmonella* included a major class that was transport negative for sulfate but retained the ability to bind the sulfate ion externally (4, 11). This binding ability for sulfate was shown to be due to a specific membrane protein which appears to be an integral part of the complete sulfate transport system of *Salmonella* (12, 13). It appears that this bacterium possesses only a single transport system for sulfate, the function of which depends on several genetic loci (4). Further work may disclose additional genetic loci in *Neurospora* that affect sulfate transport, perhaps revealing some genes that contribute an essential component to the distinct permease species reported here. Only a few genetic studies of other transport systems in *Neurospora* have been accomplished; these studies include the uptake of amino acids (5–7, 14), potassium ions (15), and sucrose (8). It appears that relatively specific transport systems are present in *Neurospora*, except that a group of related metabolites, such as the amino acids, may share transport systems and show overlapping specificities.

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