ISOLATION, CHARACTERIZATION, AND GENETIC ANALYSIS OF OSMOTIC MUTANTS OF *NEUROSPORA CRASSA*¹

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OSMOTIC (*os*) mutants of *N. crassa* are those whose growth is inhibited by high osmolalities. The usual operational definition is inhibition of growth of the mutant strains by the addition of 4% NaCl to the growth medium (PERKINS 1959). Prior to the present investigation, a group of *os* mutants had been mapped on linkage group IR, at approximately 90 map units from the centromere (PERKINS 1959; ST. LAWRENCE 1956; EMERSON 1963). These mutants will be designated *os-1*. Another mutant, called *cut*, had been mapped on the same linkage group at about 8.5 map units from the centromere (KUWANA 1960) and was shown to be inhibited by high concentrations of sucrose added to the growth medium (KUWANA 1953).

This paper presents data defining three other *os* loci on linkage group I and characterizing *cut* and *flame-2* (*flm-2*) as additional osmotic loci on linkage group I.

MATERIALS AND METHODS

Isolation of mutants: Mutants were selected by two types of techniques. One, filtration concentration (WOODWARD, DE ZEEUW and SRB 1954), depended upon the inhibition of germination of conidia from osmotic mutants by 3% NaCl. Conidia of St. Lawrence wild type 74A (ST 74A) were irradiated with ultraviolet light for 3 min at 10 ergs/sec/mm² in quartz flasks (GILES 1951). 50% of the conidia survived. Filtration of the germinated conidia from 3% NaCl minimal medium was continued for 72 hr before plating on sorbose minimal medium (DE SERRES, KÖLMARK and BROCKMAN 1962).

The second selection technique employed the properties of osmotic, *biscuit* double mutants. SRB (personal communication) had noticed that, while *biscuit* (*peak*) mutants exhibited colonial morphology, semicolonial "revertants" of such mutants frequently contained the original mutation plus a new osmotic mutation. Consequently, the selection of semicolonial colonies from a *bis* strain after mutagenesis provides an enrichment for the osmotic phenotype.

The strain used was a stock of *bis* (FGSC 277) which had been crossed to ST 74A and reisolated three times. Conidia were treated with N-methyl-N'-nitro-N-nitrosoguanidine (NNG) at a concentration of 25 µg/ml for one hour. This procedure killed 80% of the conidia, and only 7% of the original conidia remained prototrophic.

Several of the strains used in the studies reported here were isolated, by other workers, on the basis of morphological identification. These mutants, as well as ones isolated for these experiments by the means just described, are tabulated with relevant mutagen and genetic background data in Table 1. Mutants which did not cross well were not studied further, and do not appear in Table 1.

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TABLE 1

Osmotic strains analyzed in this study

Strain*	Origin	Mutagen†	Genetic background§	Linkage group
os-1 B135	FGSC‡	UV	ST × ST4 (× ST7)	IR
22	Mary Case	EMS	ST	
A11	salt filtration	UV	ST	
522	semicolonial	NNG	ST	
A46	salt filtration	UV	ST	
A51	salt filtration	UV	ST	
LO	L. Larison	UV	ST	
P668	FGSC	Spont.	M × M	
M16	FGSC	UV	M × ST3 (× ST4)	
E11200	FGSC	unknown	M	
NM233t	Perkins	UV	E × ST2 (× ST4)	
os-2 ALS-10	A. Schroeder	Mutator gene	OR	IV
os-3 S1	salt filtration	Spont.	ST	IR
S2	salt filtration	Spont.	ST	
os-4 NM201o	Perkins	?	? (× ST7)	IL
L5	salt filtration	UV	ST	
A70	salt filtration	UV	ST	
A71	salt filtration	UV	ST	
os-5 NM216o	Perkins	?	? (× ST7)	IR
C24	semicolonial	NNG	ST	
cut 176	FGSC	UV	A? × M (× ST7)	IR
A492	salt filtration	UV	ST	
A52	salt filtration	UV	ST	
A49	salt filtration	UV	ST	
flm-2 Y256M223	FGSC	UV	ST (× ST)	IR

* os = osmotic flm = flame.

† FGSC = Fungal Genetics Stock Center.

‡ UV = ultraviolet EMS = ethyl methanesulfonate NNG = N-methyl-N'-nitro-N-nitrosoguanidine Spont. = spontaneous.

§ ST = St. Lawrence M = mixed E = Emerson OR = Oak Ridge A = Abbott (× ST7) = crossed to ST74A wild type seven times before use.

Crossing: It was discovered that most of the mutants crossed very poorly on Westergaard's synthetic crossing medium (WESTERGAARD and MITCHELL 1947) but crossed better on cornmeal agar.

An additional problem encountered in the genetic analysis of these mutations was that the viability of the ascospores in crosses to wild type or with other mutants was frequently of the order of 1%, a level which essentially vitiated the usefulness of the data obtained. The viability of the ascospores was increased to levels approaching 100% by modifying the heat shocking procedure. The spores were heat shocked in agar medium containing glucose and fructose, then overplated on sorbose medium, which allowed the ascospores to be counted easily with a dissecting microscope to give direct viability data.

Complementation: The method ordinarily used to test complementation in *N. crassa* is determination of whether or not a viable heterocaryon forms when compatible strains of the same mating type are mixed under conditions which allow the growth of neither single strain. This method was considered unsuitable for this system, since the presence of a high salt concentration might well have inhibited heterocaryon formation altogether. Accordingly, osmotic strains containing complementing markers at the *pan-2* locus (*pan-2-36* and *pan-2-23*), which were

assumed to maintain a one-to-one nuclear ratio in heterocaryons (MITCHELL and MITCHELL 1950) were prepared. These strains were tested for compatibility with the *pan-2* parental strains, and then used to force the formation of heterocaryons between different osmotic strains under conditions which were nonselective for the osmotic markers. The preformed heterocaryons were then transferred to high salt medium, and their growth observed. The one-to-one nuclear ratios of the heterocaryons were confirmed.

It was discovered that *pan-2*; osmotic strains, which were heterocaryon-compatible with the complementary *pan-2* strain, were not consistently compatible with other *pan-2*; osmotic isolates. As a result, some of the *pan-2*; osmotic isolates from a cross of *pan-2* $\times$ *osmotic* appeared dominant in heterocaryons while other isolates appeared recessive. To eliminate this difficulty, several isolates of each mutant appearing "dominant" were tested. No strains were found which showed this characteristic in all isolates tested.

After the general nature of the complementation patterns had been worked out using the *pan-2*; osmotic strains as described above, it was discovered that the patterns of complementation could be duplicated using high salt concentrations (6% salt agar, plus pantothenic acid) to force the formation of heterocaryons from pairs of osmotic or *pan-2*; osmotic strains. None of the data presented in Table 8, however, with the exception of those for *os-2* ALS-10, relies on this method alone. The nuclear ratios obtained under these conditions have not been investigated.

Growth of strains for testing osmotic inhibition: When grown in small test tubes of Fries medium containing 4% NaCl, all of the strains used in this study, with the exception of ST 74A wild type, either failed to grow at all or else germinated and failed to form a mycelial mat. In order to obtain a more quantitative estimate of the osmotic sensitivities of the strains, dry weights of the mycelium produced after five days of growth at 25° in standing Erlenmeyer flasks containing 20 ml of test medium were obtained. Flasks were swirled once on the fourth day to prevent conidiation. Mycelial pads were collected on a Buchner funnel, washed with distilled water, and dried at 90°C to constant weight. The osmotic nature of the effect was checked for at least one strain at each locus by means of increasing the osmolality of the medium with KCl, NaCl, and glucose under the same conditions.

RESULTS

Selection of mutants: The frequency of recovered spontaneous osmotic mutants following filtration from 3% NaCl medium was 1×10^{-7} , and the UV-induced frequency was 4.1×10^{-6} . A 40-fold increase was thus obtained (Table 2). The frequency of semicolonial, salt-sensitive cells in the NNG-treated *bis* mutant conidial suspension was 1×10^{-5} , while in the untreated suspension the frequency was less than 2.5×10^{-7} . Part of the treated suspension was plated on glycerol complete medium; the mutation frequency calculated from these plates was 3.5×10^{-5} (Table 2).

Genetics: Allelism of the osmotic strains was established genetically by means of two-point crosses between the different osmotic strains in Table 1. The data are presented as four categories of segregants for crosses between nonallelic strains, and as parental and disomic classes for crosses between allelic strains. The apparent percent recombination was calculated for each cross as follows. Twice the number of wild-type segregants was divided by (the number of parental segregants plus twice the number of wild type segregants) and the fraction was multiplied by 100. This method was chosen for consistency, since inviability of the double mutant class was complete in some crosses (Table 3). The cause of this inviability was not determined.

Crosses yielding greater than 10% apparent recombination (Table 3) were not

TABLE 2

Selection of osmotic mutants

Technique	Mutagen	Percent survival	Frequency of mutations	Number of colonies analyzed	Number of salt-sensitive mutants
Filtration	UV	50	4.1×10^{-6}	1225	82
	Spont.	100	1.0×10^{-7}	1003	2
Semicolonial (from biscuit background)	NNG	20	1.0×10^{-5} (minimal)	2517	43
			3.5×10^{-5} (complete)	50	7
	Spont.	100	$<2.5 \times 10^{-7}$	0	0

TABLE 3

Recombination percentages between nonallelic osmotic strains

Cross P1 × P2	Progeny				Apparent percent recombination $\frac{100 \times 2 \text{ wild type}}{\text{parental} + 2 \text{ wild type}}$	Percent viability
	P1	P2	wild†	double mutant		
* <i>os-1 A11</i> × <i>os-4 NM201o</i>	32	20	23	25	46	96
* <i>os-1 B135</i> × <i>os-3 S1</i>	83	81	76	0	45	75
* <i>os-1 A11</i> × <i>os-5 NM216o</i>	36	37	13	12	25	100
<i>os-1 522</i> × <i>os-5 NM216o</i>	51	47	9	5	16	90
* <i>os-1 B135</i> × <i>os-5 NM216o</i>	21	22	5	0	19	not determined
<i>os-1 B135</i> × <i>cut A52</i>		69	8	1	19	
<i>os-1 22</i> × <i>cut 176</i>	34	34	17	14	33	95
<i>os-1 A46</i> × <i>cut 176</i>	28	27	12	11	29	85
* <i>os-1 A11</i> × <i>cut 176</i>	42	38	22	16	35	100
<i>os-1 B135</i> × <i>cut 176</i>	49	16	10	0	17	60
<i>os-1 22</i> × <i>flm-2</i>		82	18	21	29	76
* <i>os-1 B135</i> × <i>flm-2</i>	50	48	16	0	29	70
* <i>os-3 S2</i> × <i>os-4 NM201o</i>	23	22	3	3	12	100
<i>os-3 S1</i> × <i>os-4 A70</i>	33	35	18	3	29	78
<i>os-3 S1</i> × <i>os-4 A71</i>	38	40	13	8	26	96
* <i>os-3 S2</i> × <i>cut 176</i>	45	46	9	6	17	91
<i>os-3 S1</i> × <i>cut A52</i>	41	42	8	5	16	90
* <i>os-3 S1</i> × <i>os-5 C24</i>	27	26	7	0	21	93
* <i>os-3 S1</i> × <i>flm-2</i>	27	25	24	24	48	91
* <i>os-4 NM201o</i> × <i>cut 176</i>	33	33	18	19	35	83
* <i>os-4 L5</i> × <i>cut 176</i>	27	27	13	11	33	82
<i>os-4 NM201o</i> × <i>cut A52</i>	46	47	6	1	11	98
* <i>os-4 A70</i> × <i>os-5 NM216o</i>	36	35	15	4	29	97
<i>os-4 NM201o</i> × <i>os-5 C24</i>	31	29	17	3	24	82
* <i>os-4 L5</i> × <i>flm-2</i>	23	22	30	25	55	97
* <i>os-5 C24</i> × <i>cut 176</i>	44	51	5	0	10	99
* <i>os-5 NM216o</i> × <i>flm-2</i>	33	36	28	0	47	74
* <i>cut A52</i> × <i>flm-2</i>	26	27	25	22	47	93

* = Parental strains verified by complementation.

† = No corrections for pseudowild types were made.

tested for the presence of pseudowild types among the apparent wild-type segregants. The parental characters in these crosses were scored on the basis of morphological and/or salt sensitivity differences between the parental strains. This scoring procedure was verified for the crosses starred in Table 3 by sampling five isolates from each parental class and testing these isolates by complementation with both mating types of each parental osmotic marker. In all cases, the scoring appeared to be valid by this criterion.

Where five or fewer presumed double mutants were isolated from a cross between apparent non-alleles, all such isolates were tested for complementation with both parents. A sample of five isolates of this class was tested where more than five presumed double mutants were obtained. Most of these isolates were, in fact, double mutants. One presumed double mutant isolate from each of two crosses between nonallelic strains complemented with both parental strains. These two isolates were probably bisexual disomics of a type which will be discussed below.

Crosses showing less than 10% apparent recombination (Table 4) were tested for the presence of pseudowild types (caused by failure of segregation of com-

TABLE 4
Recombination analysis of allelic osmotic mutants

Cross P1 × P2	Progeny				Apparent percent recombination	Percent viability
	Parentals		Disomics*			
	P1	P2	wild type	double mutant		
<i>os-1 B135</i> × <i>os-1 22</i>	46	33	0	0	<0.6	80
	49	33	0	0		
<i>os-1 B135</i> × <i>os-1 A11</i>	56	56	0	0	<0.9	100
<i>os-1 B135</i> × <i>os-1 522</i>	49	51	0	0	<1.0	99
<i>os-1 B135</i> × <i>os-1 A46</i>		95	2	0	<0.8	95
<i>os-1 B135</i> × <i>os-1 A51</i>	26	25	0	1	<2.0	90
		250	3	2	<0.4	96
<i>os-1 B135</i> × <i>os-1 LO</i>		100	0	0	<1.0	100
<i>os-1 B135</i> × <i>os-1 P668</i>		100	0	0	<1.0	91
<i>os-1 B135</i> × <i>os-1 M16</i>		100	0	0	<1.0	94
<i>os-1 B135</i> × <i>os-1 E11200</i>		100	0	0	<1.0	90
<i>os-1 B135</i> × <i>os-1 NM233t</i>		100	0	0	<1.0	95
<i>os-3 S1</i> × <i>os-3 S2</i>		99	0	0	<1.0	85
<i>os-4 NM201</i> × <i>os-4 L5</i>	44	44	0	0	<0.6	95
	49	51	0	0		
<i>os-4 NM201</i> × <i>os-4 A70</i>	25	143	3	0	<0.6	47
<i>os-4 NM201</i> × <i>os-4 A71</i>	49	48	3	0	<1.0	92
<i>os-5 NM216</i> × <i>os-5 C24</i>		100	0	0	<1.0	92
<i>cut 176</i> × <i>cut M1</i>	48	51	0	0	<1.0	87
<i>cut 176</i> × <i>but A492</i>	49	48	2	0	<1.0	81
<i>cut 176</i> × <i>cut A52</i>	49	51	1	0	<1.0	99
<i>cut 176</i> × <i>cut A49</i>	47	53	2	0	<1.0	93

* See text for explanation.

plementing mutations because of nondisjunction (PITTINGER 1954)) among the apparent wild-type segregants, since such pseudowild types could obscure the allelism of complementing mutants at a given locus. Conidia of apparent wild-type segregants were suspended and plated at 30 to 40 colonies per plate: 50 colonies were then picked and tested for salt sensitivity; if none was sensitive, the original isolate was inferred to be wild type instead of pseudowild type. Those isolates which yielded salt-sensitive segregants showed at least 10% of such segregants, and thus were designated as pseudowild types. The apparent double mutants from these crosses between presumed alleles were separated from the parental classes on the basis of more abnormal colony morphology, and tested as follows. About 50 colonies were picked from plates of each of these isolates and tested for complementation with the parental types. Heterocaryotic isolates should have yielded some colonies able to complement with both parental classes, whereas double mutants should have produced only colonies able to complement with neither parental type. In fact, all of the colonies from these isolates complemented with either one or the other of the parental types, but not both, which suggested that the original isolate in each case had been a disomic type whose heterocaryon had virtually completely broken down. This behavior is characteristic of disomics of linkage group I in *N. crassa* which contain both alleles of the mating-type locus (NEWMAYER and TAYLOR 1967), but these isolates occurred at a higher frequency than the known disomics. Since the mating types of the complementing parental markers were not recorded in the present investigation, it was not shown that these isolates were in fact bisexual. They were not, however, stable heterocaryons, single parentals, double mutants, or wild types. The mixed conidia of the original isolate should be able to complement with appropriate mating types of both parental strains; thus, these strains were probably similar to those cited earlier in the apparent double mutant classes which complemented with both parental strains. It should be noted that these isolates could conceivably have arisen from two ascospores, although the method of isolation makes this possibility unlikely.

Since two-point crossing data from *N. crassa* cannot always be relied upon for distances to be used in the ordering of markers (PERKINS 1959), the orders obtained from the two-point data were checked by means of three-point crosses. Strains bearing two markers near the suspected locus of the osmotic group involved were crossed to a mutant of the locus, and the relative order of the three markers determined (Table 5).

The two-point crossing data are summarized in Table 4 and in the lower portion of Figure 1. All of the strains studied in this investigation map on linkage group I at six different locations.

Os-3, the only locus for which there exist neither published nor unpublished map data other than those presented here, was the only type of spontaneous mutant recovered from filtration concentration. The two strains were isolated from different plates and have rather different cell walls (LIVINGSTON 1968) so the two are not likely to be two isolates from the same mutational event.

The data on which locus groupings are based are given in Table 4. Pseudowild

TABLE 5

Three-point mapping analysis of osmotic loci

<div> <div>Cross*</div> <div> <div>P1</div> <div>P2</div> </div> </div>	Progeny							Coefficient of coincidence	Percent viability
	Parentals		Crossovers						
			Single		Region 2		Double		
P1	P2	Region 1		Region 2		Double			
<div> <div> <div>+ 6 os-4 14 +</div> <div>leu-3 + arg-1</div> </div> <div>○</div> </div>	23	17	2	1	3	4	0	1.00	78
<div> <div> <div>+ 40 + 24flm-2</div> <div>al-2 os-1 +</div> </div> <div>○</div> </div>	19	22	18	17	10	9	5	0.52	99
<div> <div> <div>+ 3 + 10 os-5</div> <div>arg-6 al-2 +</div> </div> <div>○</div> </div>	94	80	4	2	12	8	0	1.00	93
<div> <div> <div>cut 12 + 2 +</div> <div>+ arg-6 al-2</div> </div> <div>○</div> </div>	..	..	..	..	..	..	..	..	..
<div> <div> <div>+ 10 + 4 os-3</div> <div>arg-1 nic-2 +</div> </div> <div>○</div> </div>	85	88	9	10	3	3	1	1.25	98

* Cross parents: Map units indicated in bold face; direction of centromere indicated by circle.

1. FGSC 1216: *leu-3* B269, *arg-1* R156a × *os-4* L5 A.

2. FGSC 866: *al-2* 15300, *os-1* P641A × *flm-2*, *pan-2-23a*.

3. FGSC 313: *arg-6* 29997, *al-2* 1530CA × *os-5* C24a.

4. FGSC 272: *arg-6* 29997, *al-2* 15300a × *cut* A492, *pan-2-36A*.

5. Mary Case: *arg-1*, *nic-2a* × *os-3* S1, *pan-2-36A*.

formation occurs in crosses between pairs of strains at each locus at which more than two strains have been mapped (Table 4). The figure chosen for locus definition, namely, less than 1% recombination, is probably not low enough to exclude any complex region of several loci having similar functions. Some of these groups, therefore, may be clusters of loci rather than single loci.

The loci whose positions appeared uncertain, i.e., *os-4*, *os-3*, *cut* and *flm-2*, were ordered by means of three-point crosses. The positions assigned agree with those postulated from two-point crosses, as can be seen from Table 5. The map of linkage group I which resulted from the reported studies is presented in the upper portion of Figure 1.

Complementation: The complementation data are presented in Table 6. The complementation groups correspond exactly with those assigned by crossing. The ± category signifies those heterocaryons which germinated when transferred to 4% NaCl medium, but which did not grow; these responses were counted as non-complementation, although they may be instances of intragenic complementation. Those complementation groups in which these responses occurred correspond to loci at which pseudowild types were found to occur (Tables 4 and 6). The pairs in question, however, did not always form pseudowild types when a ± complementation response was found. Perhaps the pseudowild formation was below the level detectable by isolation of about 100 progeny, in some cases. Levels of

FIGURE 1.—Map of linkage group I showing positions of the osmotic loci. In the upper portion of the figure, markers used to map the osmotic loci are above the line and the osmotic loci are below the line. In the lower portion of the figure, map distances between the loci are shown.

pseudowild formation less than 1% have been reported (PITTENGER 1954). All of the cases which showed pseudowild formation did show a $\pm$ complementation response, suggesting that intragenic complementation is the probable explanation for this response.

Osmotic inhibition: It is noteworthy that many of the osmotic strains produced more dry weight of mycelium per 20 ml culture than wild type ST 74A in Fries medium alone. These high dry weights probably result from parallel increases in both cell wall and cytoplasmic components since the cell wall dry weights per unit mycelium dry weight for the osmotic strains were not significantly different from wild type (LIVINGSTON 1968).

The mutants which germinated but failed to grow in small tubes of 4% NaCl medium were able to form mats in flasks, but all were still inhibited to some extent by the high salt concentrations (Table 7). It is possible that the aeration is better in flasks, and that aeration interacts in some way with the osmotic pressure effect.

Some strains of all osmotic groups except *cut* and *flm-2* failed to grow at all when 0.4M NaCl was added to the medium (total activity of resultant medium = 379 milliosmolal) (Table 7). These strains were designated as completely inhibited. Strains which grew at 379 milliosmolal activity were designated as partially inhibited. Three strains, *cut* 176, *flm-2* and *os-1* A51 were not inhibited as much as wild type by this level of medium osmolality (Table 7). All strains, how-

TABLE 6
Complementation of pairwise combinations of the osmotic strains

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. B135	—																						
2. 22	—	—																					
3. A11	—	—	—																				
4. 522	+	—	+	—																			
5. A46	+	+	+	+	—																		
6. A51	+	+	+	+	+	—																	
7. LO	—	—	—	—	—	—	—																
8. M16	—	—	—	—	—	+	—	—															
9. S1	+	+	+	+	+	+	+	+	—														
10. S2	+	+	+	+	+	+	+	+	+	—													
11. NM201	+	+	+	+	+	+	+	+	+	+	—												
12. L5	+	+	+	+	+	+	+	+	+	+	+	—											
13. A70	+	+	+	+	+	+	+	+	+	+	+	+	—										
14. A71	+	+	+	+	+	+	+	+	+	+	+	+	+	—									
15. NM216	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—								
16. C24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—							
17. cut 176	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—						
18. M1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—					
19. A492	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—				
20. A52	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—			
21. A49	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—		
22. flm-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	
23. ALS-10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—

* ± = heterocaryon germinated but did not grow on 4% salt medium.
 + = heterocaryon grew more than either control on 4% salt medium.

TABLE 7

Effect of medium osmolality on growth of wild-type and osmotic strains*

Strain	Fries	mg. dry weight:† Fries plus 0.4M NaCl	Fries plus 0.8 M NaCl	Fries plus 1.2M NaCl	Designation of phenotype‡
STL 74A	80.0	75.9	55.8	28.9	
<i>os-4</i>					
NM201o	93.7	82.6	52.2	9.9	partially inhibited
L5	118.9	0.0	0.0	0.0	completely inhibited
A70	99.7	74.9	49.3	15.2	partially inhibited
A71	100.6	70.3	52.3	16.1	partially inhibited
<i>os-3</i>					
S1	55.5	11.8	0.9	0.0	partially inhibited
S2	101.2	0.0	0.0	0.0	completely inhibited
<i>cut</i>					
176	85.1	90.5	36.8	5.6	partially inhibited
A492	100.7	77.1	57.0	18.9	partially inhibited
A52	86.2	47.1	19.0	0.0	partially inhibited
A49	88.7	68.4	51.8	15.3	partially inhibited
<i>os-5</i>					
NM216o	110.3	81.8	51.1	20.8	partially inhibited
C24	106.1	0.0	0.0	0.0	completely inhibited
<i>os-1</i>					
B135	68.1	44.5	0.0	0.0	partially inhibited
E11200	91.9	0.0	0.0	0.0	completely inhibited
M16	93.5	0.0	0.0	0.0	completely inhibited
NM233t	83.4	68.4	23.0	0.0	partially inhibited
LO	94.6	0.0	0.0	0.0	completely inhibited
22	90.5	58.9	53.9	0.0	partially inhibited
522	104.3	73.5	50.4	19.3	partially inhibited
A46	82.9	45.6	35.9	0.0	partially inhibited
A51	95.4	91.9	40.8	17.3	partially inhibited
A11	101.9	79.2	33.4	0.8	partially inhibited
<i>flm-2</i>	76.4	78.2	34.5	16.9	partially inhibited

* The osmolality of Fries medium was increased by means of adding NaCl.

† Dry weights (average of two determinations) were taken after five days of growth at 25° in 20 ml of medium.

The largest variation observed between two averaged determinations was 1 mg for the data in this Table.

‡ See text for explanation.

ever, were inhibited more than wild type at 891 milliosmolal (medium plus 1.2 M NaCl). The osmotic strains from each locus which were tested with KCl and glucose additions to Fries medium (Table 8) were inhibited more than wild type by these compounds as well as by NaCl, confirming the osmotic nature of the phenotype.

All strains, therefore, appear to be osmotically sensitive, but the levels of sensitivity vary. No indication of locus specificity in the degree of osmotic sensitivity was evident, except that no mutant mapping at the *cut* locus was completely inhibited (Table 7). The lack of correlation of sensitivity with locus groups may be the result of different degrees of leakiness of the different alleles at each locus.

TABLE 8

Effect of medium osmolality† on growth of wild-type and osmotic strains

Strain	Fries	mg dry weight:*		Fries plus 2.0M glucose
		Fries plus 1.1M KCl	Fries plus 1.3M NaCl	
ST 74A	98.1	90.2	62.4	58.8
<i>os-4 L5</i>	94.8	0.0	0.0	0.0
<i>os-3 S2</i>	97.7	0.0	0.0	0.0
<i>cut 176</i>	86.6	84.5	44.8	26.8
<i>os-5 C24</i>	98.2	0.0	0.0	0.0
<i>os-1 B135</i>	88.1	0.0	0.0	0.0
<i>flm-2</i>	76.0	55.8	48.1	12.4

† The osmolality of Fries medium was increased by adding NaCl, KCl, or glucose.

• Dry weights (average of two determinations) were taken after five days of growth at 25°C in 20 ml of medium.

The largest variation between averaged determinations was 8 mg for the data in this Table.

DISCUSSION

Prior to this investigation and during it, several osmotic loci were mapped by other investigators. The *NM201o* allele of *os-4* was mapped by PERKINS (personal communication) at less than six map units from mating type in two crosses. Since mating type has been located at 12 units left of the centromere (12L) according to PERKINS (1959) and at about 6L according to KUWANA (1960), the position of *os-4* at about 12L (Figure 1) agrees with this general position near mating type.

Cut was mapped by KUWANA (1960) at 7R, which is closer to the centromere than the present data would place it (Table 3, Figure 1). Note, however, that one of the estimates of the distance between *cut* and *os-4* was 11 (Table 3), which suggests that a variable interference in the centromere region might be occurring. Such an explanation is supported by the disparate distances from the centromere of the *crisp* alleles mapped by GARNJOBST and TATUM (1967). BOLE-GOWDA, PERKINS and STRICKLAND (1967), however, found little or no interference across the centromere of linkage group I. The positive interference, then, may be due to contributions of the regions of the left and right arms separating these markers. PERKINS has indicated (personal communication) that the original *cut* stock had a reciprocal translocation between linkage groups I and IV; however, this translocation appears to have been lost during the inbreeding of the stock into St. Lawrence wild-type background.

The *os-5* stock *NM-216o* was mapped by PERKINS (personal communication) at about three map units from *albino-2*, which has been located at about 40R (KUWANA 1960) and 50R (PERKINS 1959). This position agrees with the location at 41R to which *os-5* was assigned in Figure 1.

Os-1 B135 has been mapped by PERKINS (1959) at around 80R, and by KUWANA (1960) at around 52R, whereas *os-1* appears to be at about 66R from the present two-point data (lower portion of Figure 1). The distance between *cut* and *os-1* which KUWANA (1960) obtained was 41 map units, which is slightly higher

than any value obtained in these studies (Table 3, Figure 1) but in reasonable agreement with the highest value, 35 map units. Such differences in map data may either be due to differences in genetic background or to sampling error (PERKINS 1959). In addition, where viability was poor and widely different allele ratios were obtained, selection could account for part of the variability (PERKINS 1959).

The Fungal Genetics Stock Center (FGSC) lists *flame-2* (*flm-2*) on linkage group I. On the basis of the present results, *flm-2* appears to be beyond *os-1* at approximately 95R.

Os-2 (*ALS-10*) was isolated by SCHROEDER, and found to be linked to *cot-1* at about 4 units away, on the side opposite *iv-3* (*Y 7110*) (SCHROEDER, personal communication).

The location of all six osmotic loci on linkage group I raises the question of possible functional interaction between the loci. While they are rather far apart, based on the distances observed, and apparently unrelated loci are known to occur between them (Figure 1), there is a possibility that the entire region in which they occur functions as a unit in the developmental processes of the mold. Those morphological mutations which have been mapped to date tend to occur more frequently on linkage group I than on other linkage groups, but many have not yet been located since they are not linked to any known marker. It has been suggested that the large number of multiply marked genetic tester stocks which are available for this linkage group, coupled with the comparatively long genetic length of this linkage group, might cause the data to suggest a concentration of morphological mutants in linkage group I even if such mutants were evenly distributed throughout the whole genome (GARNJOBST and TATUM 1967).

Since extensive crosses have not been made among the individual mutants mapping within each locus, the possibility cannot yet be eliminated that some of the apparent osmotic loci are in fact clusters of genes similar to the gene cluster for the *arom* enzyme complex in *N. crassa* (GILES *et al.* 1967).

The results of complementation studies among these loci presently suggest a straightforward relationship involving free interaction of the various gene products. Complementation between mutants at different loci always occurred, after the pseudodominance phenomenon was discovered and adequately controlled. These results are most easily explained by assuming that osmotic mutations affect internal metabolism so as to produce an imbalance in the supply of precursors to the cell wall synthesizing enzymes. In fact, these strains do have definite but quantitatively small changes in their overall cell wall compositions (LIVINGSTON 1969), as well as in their gross appearance in the electron microscope (LIVINGSTON 1968). The recessive nature of the phenotype tends to rule out a model involving a rampant cell wall autolytic enzyme (as proposed by HAMILTON and CALVET 1964) no longer responsive to the usual controls. The results also cast some doubt on a model that these loci specify a multifunctional complex of cell wall synthesizing enzymes which interact structurally, on the membrane or elsewhere, since some incidence of noncomplementation between mutants at the different loci would be predicted from such a model. It is still possible that the latter

model is correct, however, since protein-protein interactions might not have been affected in any of the mutant strains studied.

Finally, it is possible that a change in the membrane structure which only mildly affects cell wall synthesis has occurred. Such disturbance in cell wall metabolism might result from a slight conformational disturbance of the cell wall synthesizing enzymes, or from a change in the transport capabilities of the osmotic strains.

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SUMMARY

Neurospora crassa mutants of the osmotic phenotype characterized by inhibition of growth at high osmolalities of growth medium were isolated by filtration concentration from 3% NaCl medium and by selecting semicolonial isolates of a colonial mutant. From a total of 132 salt-sensitive mutants isolated, 14 mutants which crossed well were studied in detail. In addition, nine osmotic strains isolated by other investigators were studied. All of the osmotic mutants mapped at one of six loci on linkage group I. The positions of these six loci, based on two-point crossing data, are *os-4* at 12L, *os-3* at 15R, *cut* at 31R, *os-5* at 41R, *os-1* at 66R, and *flm-2* at 95R. The positions of all of the loci except *os-1* were verified by means of three-point crosses with known linkage group I markers. Complementation patterns, obtained using forced heterocaryons, corresponded to the genetically defined loci. There was some evidence for weak intragenic complementation at four of the six loci studied. The osmotic phenotype was recessive in all cases. The strains were characterized as to their levels of osmotic sensitivity, but no obvious correlations between these levels and the various osmotic loci were found.

LITERATURE CITED

- BOLE-GOWDA, B., D. PERKINS and W. N. STRICKLAND, 1962 Crossing over and interference in the centromere region of Linkage Group I of *Neurospora*. *Genetics* **47**: 1243-1252.
- DESERRES, F., H. G. KØLMARK and H. E. BROCKMAN, 1962 Factors influencing the survival of *Neurospora crassa* conidia in sorbose media. *Nature* **193**: 556-557.
- EMERSON, S., 1963 Slime: A plasmodioid variant of *Neurospora crassa*. *Genetics* **34**: 162-182.
- GARNJOBST, L. and E. L. TATUM, 1967 A survey of new morphological mutants in *Neurospora crassa*. *Genetics* **57**: 579-604.
- GILES, N. H., 1951 Studies on the mechanism of reversion in biochemical mutants of *Neurospora crassa*. Cold Spring Harbor Symp. Quant. Biol. **16**: 283-313.
- GILES, N. H., M. E. CASE, C. W. H. PARTRIDGE and S. I. AHMED, 1967 A gene cluster in *Neurospora crassa* coding for an aggregate of five aromatic synthetic enzymes. *Proc. Natl. Acad. Sci. U.S.* **58**: 1453-1460.
- HAMILTON, J. G. and J. CALVET, 1964 Production of protoplasts in an osmotic mutant of *Neurospora crassa* without added enzyme. *J. Bacteriol.* **88**: 1084-1086.

- KUWANA, H., 1953 Studies on the morphological mutant "cut" in *Neurospora crassa*. *Cytologia* **18**: 235-239.
- KUWANA, H., 1960 Studies on a temperature-sensitive irreparable mutant of *Neurospora crassa*. *Japan J. Genetics* **35**: 49-57.
- LIVINGSTON, L. R., 1968 The genetics and cell surface characteristics of the osmotic phenotype of *Neurospora crassa*. Ph.D. dissertation, Yale University.
- LIVINGSTON, L. R., 1969 Locus-specific changes in cell wall composition characteristic of osmotic mutants of *Neurospora crassa*. *J. Bacteriol.* **99**: 85-90.
- MITCHELL, M. B. and H. K. MITCHELL, 1950 The selective advantage of an adenineless double mutant over one of the single mutants involved. *Proc. Natl. Acad. Sci. U.S.* **36**: 115-119.
- NEWMAYER, D. and C. TAYLOR, 1967 A pericentric inversion in *Neurospora* with unstable duplication progeny. *Genetics* **56**: 771-791.
- PERKINS, D. D., 1959 New markers and multiple point linkage data in *Neurospora*. *Genetics* **44**: 1185-1208.
- PITTENGER, T., 1954 The general incidence of pseudo-wild types in *Neurospora crassa*. *Genetics* **39**: 326-342.
- ST. LAWRENCE, P., 1956 The *g* locus of *Neurospora crassa*. *Proc. Natl. Acad. Sci. U.S.* **42**: 189-194.
- WESTERGAARD, M. and H. K. MITCHELL, 1947 *Neurospora*. V. A synthetic medium favoring sexual reproduction. *Am. J. Botany* **34**: 573-577.
- WOODWARD, V., J. R. DE ZEEUW and A. M. SRB, 1954 The separation and isolation of particular biochemical mutants of *Neurospora* by differential germination of conidia, followed by filtration and plating. *Proc. Natl. Acad. Sci. U.S.* **40**: 192-200.