Genetic Analysis of the Slug Stage of *Dictyostelium discoideum*

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During the course of development, cells of *Dictyostelium discoideum* are able to produce a multicellular body (a 'slug') which is capable of movement over the substratum. This phase, which is induced by production of ammonia by the starving cells, may last for hours or days depending on environmental stimuli. In order to probe the regulatory system controlling formation and duration of the slug phase, mutants were isolated that remained in the slug phase for an extended period. Thirty-two such 'slugger' mutants were analysed by parasexual genetic techniques and placed into 10 complementation groups (*slgA*–*slgJ*). The linkage groups bearing representatives of these complementation groups were determined by segregation of diploids formed between mutants and tester strains. Phenotypic studies of mutants indicated that members of *slgD*, *slgE* and *slgG* were over-sensitive to the ammonia slug-inducing stimulus.

INTRODUCTION

The normal pathway of development of the cellular slime mould involves the formation of a migratory phase in which the multicellular aggregate is able to move over the substratum with a speed of approximately 1 mm h^{-1} . This 'slug' phase lasts for a variable length of time (hours or days) depending on the environmental stimuli (Slifkin & Bonner, 1952; Newell *et al.*, 1969) and probably has ecological significance in ensuring that fruiting body construction occurs at the surface of the substratum rather than underneath decaying leaves in the natural habitat of forest litter. From studies examining the pattern of formation of several developmentally regulated enzymes (UDPglucose pyrophosphorylase, UDPgalactose epimerase, UDPgalactose transferase and trehalose phosphate synthetase), it appears that while the slug migrates the normal programme of events seen during fruiting body construction is held in abeyance (Newell & Sussman, 1970; Newell *et al.*, 1972). The stimulus to produce the slug stage is a basic volatile metabolite produced by the aggregating amoebae (Newell *et al.*, 1969) that has been identified by Schindler & Sussman (1977) as ammonia. Movement of the slug is by production of a slime sheath at the front of the slug to form an extended tube, like a sausage skin, through which the amoebal aggregate moves, leaving a collapsed and empty skin or 'slime trail' behind it (Raper, 1940). The migrating slug responds to light and heat signals of very low intensity, moving towards a light source parallel to the substratum and up a heat gradient of only $0.05 \text{ }^{\circ}\text{C cm}^{-1}$ (Bonner *et al.*, 1950; Francis, 1964; Poff & Skokut, 1977). Recent experiments of Fisher *et al.* (1981) suggest that the operation of these responses requires the production of a factor (called STF) that causes the tip to turn in response to the stimuli. Mutants have been found (Loomis, 1970) which are unable to respond to light signals and which migrate at random in a light gradient. The nature of the light receptor has been extensively studied by Poff and coworkers (Poff *et al.*, 1974; Manabe & Poff, 1978) but has not yet been rigorously identified. Environmental factors that halt slug migration and restart the development of the fruiting body include a drop in humidity (Slifkin & Bonner, 1952), the

presence of phosphate buffer at a pH of 6.0–6.5 and overhead light (Newell *et al.*, 1969). The biochemical mechanism of action of these stimuli is unknown but is thought to involve the production of a factor (appropriately termed 'fruit juice') that has the properties of an aliphatic acid and which may be mimicked by sodium propionate (Sussman *et al.*, 1978).

The formation and termination of the slug phase in *Dictyostelium discoideum* is an important example of a regulatory system involving the behaviour of a multicellular structure. One approach to understanding this phenomenon is to isolate mutants that are defective in various aspects of the system and to determine the biochemical nature of their lesions. In the study described below, mutants (designated 'sluggers') were isolated which are able to form slugs but are unable to revert to the programme for forming the fruiting body (or revert only after an excessively long period of slug migration). Prior to this study one such mutant (designated KY3) had been reported (Yanagisawa *et al.*, 1967) and this was included in the genetic analysis together with two other slugger mutants (JC2 and JC4) isolated more recently (Sussman *et al.*, 1978).

METHODS

Media and chemicals. The composition and methods of preparation of nutrient (SM), KK₂ and drug selective media, salts solution (SS) and chemicals were as described previously (Mosses *et al.*, 1975; Ross & Newell, 1979, 1981; Wallace & Newell, 1982). The composition of Lower Pad Solution (LPS) is that of Newell *et al.* (1969).

Growth conditions. All strains of *Dictyostelium discoideum* were grown in the dark in association with *Klebsiella aerogenes* at 22 °C, or at the restrictive temperature (27 °C). *Bacillus subtilis* strain 36.1, which was used for the selection of *D. discoideum* diploids and for testing for the presence of the *bsg* markers, has been described previously (Newell *et al.*, 1977a).

Strains. The origins and genotypes of haploid strains are given in Table 1. The strains are all derivatives of NC4. The nomenclature system used is based on that of Demerec *et al.* (1966). Primary stocks were maintained as spores, either as pure cultures or as synergistic mixtures of mutant and wild-type. Spores were collected in horse serum, dried on silica gel and stored at 4 °C (Perkins, 1962). Mutants were also stored frozen as amoebae in horse serum containing 5% (v/v) dimethyl sulphoxide in plastic straws immersed in liquid nitrogen (Williams & Newell, 1976). Cloning of stocks by 'streak cloning' and by 'spreader dilution' has been described previously (Wallace & Newell, 1982).

Formation of diploids and segregation of haploids. Diploid strains were obtained by shaking amoebae of haploid strains bearing non-allelic diploid-selection markers such as *bsgA5* and *tsgK21* and selecting with *B. subtilis* at 27 °C as described by Newell *et al.* (1977a, b). Ploidy was determined on the basis of spore size (diploids being larger) or, where no spores were formed, by cytological staining using the rapid method of Welker & Williams (1980c). Diploids were haploidized for segregation analysis using selective drug resistance markers as described by Williams *et al.* (1974). During the later stages of this study the improved haploidization method of Wallace & Newell (1982) was used, as this method reduces the tendency of clonal growth effects to bias the segregation pattern. Where suitable drug resistance markers were not available the haploidizing agent benlate was employed (Williams & Barrand, 1978).

Mutagenesis and isolation of slugger mutants. Freshly harvested amoebae were incubated at 10⁷ ml⁻¹ in SS salts solution for 10–45 min with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 200 or 500 µg ml⁻¹ so as to give approximately 10% survival. Mutagenized amoebae were washed three times in SS, plated on KK₂ plates without bacteria and allowed to develop. The resulting spores (approximately 5 × 10⁶ per plate) were harvested and stored in 80% (v/v) glycerol at -20 °C. Screening for mutants was carried out by plating spores on SM agar at about 50 spores per plate with an inoculum of *K. aerogenes* and allowing growth of clones to approximately 20 mm diameter. Clones failing to develop beyond the slug stage were retained, recloned and retested. Because one division, at most, could have occurred during the development of the mutagenized amoebae on the KK₂ plates, it is very unlikely that any of the mutants obtained by this technique had a common origin. Because slugger mutants synergize freely, the technique of allowing sporulation before screening for mutants did not prevent isolation of slugger mutants despite their being unable to sporulate in clonal culture. To ensure that classes of slugger mutants that were unable to sporulate by synergism were not missed by the isolation procedure, some experiments employed plating and screening on the SM agar directly after mutagenesis of the amoebae.

Determination of slugger phenotype. For genetic experiments, the slugger phenotype was scored by observing the formation of slugs (rather than mature fruiting bodies) in colonies of 10–20 mm diameter. The colonies were initiated by transfer of amoebae by sterile toothpick on to SM agar inoculated with *K. aerogenes* and grown at 22 °C in the dark. For comparison of developmental timing, washed amoebae from SM growth plates were

Table 1. Genotypes of parental haploid and multiply marked tester strains of *D. discoideum* used

Mutant phenotype corresponding to each genotype symbol: *cyc*, resistance to cycloheximide (500 µg ml⁻¹); *spr*, round spores; *whi*, white spore mass; *acr*, resistance to methanol (2%, v/v); *axe*, ability to grow in axenic medium; *tsg*, temperature-sensitive for growth; *bsg*, unable to grow on *Bacillus subtilis*; *bwn*, production of brown pigment; *ebr*, resistance to ethidium bromide (35 µg ml⁻¹); *man*, lack of α-mannosidase; *cob*, resistance to CoCl₂ (300 µg ml⁻¹).

Strain	Parent(s)	<i>cyc</i>	<i>spr</i>	<i>whi</i>	<i>acr</i>	<i>axe</i>	<i>tsg</i>	<i>bsg</i>	<i>bwn</i>	<i>ebr</i>	<i>man</i>	<i>cob</i>	Ref.*
X22	NP14, M28	.	<i>AI</i>	<i>AI</i>	<i>AI</i>	.	<i>D12, E13</i>	.	.	.	.	.	<i>a</i>
XP55	NP194, X22	<i>A5</i>	.	.	.	.	<i>K21</i>	<i>A5</i>	.	.	.	.	<i>b</i>
NP187	NP20	<i>A5</i>	.	.	.	.	<i>D12, E13</i>	<i>A5</i>	.	.	.	.	<i>c</i>
XP57	X22, NP194	.	<i>AI</i>	<i>AI</i>	<i>AI</i>	.	<i>D12, E13</i>	<i>A5</i>	.	.	.	.	<i>d</i>
XP64	XP58, XM101	<i>AI</i> or <i>S</i>	.	.	.	.	.	<i>A5</i>	.	.	<i>A2</i>	<i>AI</i>	<i>e</i>
XP76	X36, XM101	<i>AI</i>	.	.	.	<i>AI, B1</i>	<i>AI</i>	.	<i>AI</i>	.	<i>A2</i>	<i>AI</i>	<i>f</i>
XP86	XP64, XP71	.	.	<i>AI</i>	<i>A2</i>	.	.	<i>A5</i>	<i>AI</i>	.	<i>A2</i>	<i>AI</i>	<i>g</i>
XP95	XP64, XP71	.	.	<i>AI</i>	<i>A2</i>	<i>B1</i>	<i>AI</i>	.	<i>AI</i>	.	<i>A2</i>	<i>AI</i>	<i>e</i>
XP144	XP95, XP99	<i>AI</i> or <i>S</i>	.	<i>AI</i>	<i>A2</i>	.	.	<i>A5</i>	<i>AI</i>	.	<i>A2</i>	<i>AI</i>	<i>g</i>
XP232	XP81, X2	.	.	<i>AI</i>	<i>A2</i>	.	.	<i>A5</i>	<i>AI</i>	.	<i>A2</i>	.	<i>h</i>
HL100	NC4	.	.	.	.	.	.	<i>B500</i>	.	.	.	.	<i>i</i>
HU982	DU1342	.	.	<i>AI</i>	<i>D1817</i>	.	<i>D12</i>	<i>C350</i>	<i>AI</i>	<i>B359</i>	.	.	<i>j</i>

* References: *a*, Williams & Newell (1976); *b*, Newell *et al.* (1977*a*); *c*, Wallace & Newell (1982); *d*, Newell (unpublished); *e*, Ratner & Newell (1978); *f*, Ross & Newell (1979); *g*, Ratner & Newell (unpublished); *h*, this study; *i*, Morrissey *et al.* (1980); *j*, Williams & Welker (unpublished).

dispensed on to black Whatman no. 29 filters resting on Whatman no. 17 support pads saturated with LPS, as described by Newell *et al.* (1969). The concentration of phosphate used was 40 mM and (unless stated otherwise) no upper pads were employed.

RESULTS

Isolation of slugger mutants

The slugger phenotype may be observed in clones growing on lawns of *K. aerogenes* by the failure to complete the normal developmental programme after the slug stage. Using the parental *D. discoideum* strains X22, XP55 and NP187, 32 stable slugger mutants were isolated from eight separate mutagenesis experiments. Additionally, three other mutants (KY3, JC2 and JC4) isolated by M. Sussman in the wild-type (NC4) background were included in this study. All of these mutants were found to synergize with the parental type and stocks were kept of mixed wild-type and mutant spores formed by synergistic development. Most of the mutants formed slugs that were fully capable of migration but a few formed only upright slugs or 'fingers' as though they were blocked at the stage just before that of production of the horizontal slug form. Mutants that remained in the slug phase longer than normal but were then fully capable of forming fruiting bodies were not retained unless the duration of the slug mode was longer than 24 h.

The parental strains used for isolating the slugger mutants possessed either temperature-sensitive growth (*tsg*) or *Bacillus subtilis* sensitivity (*bsg*) mutations so that diploids could be selected when required for the subsequent genetic studies. The mutants KY3, JC2 and JC4, however, lacked any diploid selector. To enable genetic studies on these mutants, *tsg* mutations were selected after mutagenesis and screening for mutants unable to grow at the non-permissive temperature of 27 °C but able to grow at 22 °C. Although temperature-sensitive mutants normally fail to grow at the non-permissive temperature, whatever bacterial food source is used, it was noticed that in the case of strain NP389 (the *tsg* derivative of the slugger strain KY3) growth was fully temperature-sensitive on *K. aerogenes* but only partially so on *B. subtilis*.

Complementation analysis

After fusion of the mutants in pairs or with the wild-type and testing the resultant diploids for the presence of the slugger phenotype, the mutations were found to be recessive and were placed into 10 complementation groups, *slgA*–*slgJ* (Table 2).

As five of the complementation groups contained both *bsg*-bearing mutants and/or mutants with non-allelic *tsg* mutations, the majority of fusions could be carried out within and between groups without difficulty. However, in a number of cases (groups *slgF*, *slgG*, *slgH*, *slgI* and *slgJ*), in which all representatives of each group were found in the same genetic background, derivatives of the strains had to be constructed bearing other fusion markers by segregation from appropriate diploids (see Table 2). In the cases of *slgB* and *slgG* (which are linked to the *bsgA* marker on linkage group III) the required slugger derivatives lacking the *bsgA* marker could not be simply constructed by segregation. Instead, they were made by selecting haploids that contained *tsgK21*, *bsgA5*, *slgB401* (or *slgG407*) from diploids which were heterozygous for these markers, followed by reversion of the *bsgA5* mutation as described in the analogous study of streamer mutations by Ross & Newell (1979). These strains [such as NP414 (*slgB*) and NP418 (*slgG*)] were required for cross-checking the XP55-derived slugger mutants, all of which bear *bsgA5*. Analogous strain manipulations for *slgH* and *slgJ* (which are linked to *tsgK* on linkage group VII) attempting to eliminate the *tsgK21* marker by reversion were not found to be satisfactory for use with complementation studies. As reported by Wallace & Newell (1982), isolation of revertants (or suppressors) of the *tsgK21* locus using selection for growth at the non-permissive temperature very often introduced additional growth and developmental mutations by an unexplained mechanism. In

Table 2. *Complementation groups determined for the slugger mutants*

The last column shows derivative strains in which the diploid selector markers (*bsgA5* or *tsgK21*) have been exchanged for other selectors (such as *bsgB500* from strain HL100, or *bsgC350* from HU982) and, in certain cases, *bsgA5* has been reverted. Strains used most frequently to establish complementation groups are indicated by asterisks.

Complementation group	Mutant name	Mutation number	Parental strain	Representative derivatives with diploid selector in parentheses
<i>slgA</i>	*NP208	<i>slgA400</i>	NP187	*XP189 (<i>tsgD12 tsgE13 tsgK⁺</i>)
	NP419	<i>slgA409</i>	XP55	
	NP437	<i>slgA424</i>	NP187	
	NP443	<i>slgA430</i>	NP187	
	NP444	<i>slgA431</i>	NP187	
	NP446	<i>slgA433</i>	NP187	
	NP451	<i>slgA438</i>	NP187	
<i>slgB</i>	*NP347	<i>slgB401</i>	XP55	*NP414 (<i>tsgK21 bsgA⁺</i> reverted)
	NP388	<i>slgB403</i>	XP55	
	NP407	<i>slgB405</i>	XP55	
	NP445	<i>slgB432</i>	NP187	
	NP454	<i>slgB441</i>	NP187	XP243 (<i>bsgB500 tsgK⁺</i>)
<i>slgC</i>	*NP351	<i>slgC402</i>	X22	
	JC2	<i>slgC701</i>	NC4	NP396 (<i>tsg-404</i>)
	JC4	<i>slgC702</i>	NC4	NP417 (<i>tsg-406</i>)
	NP426	<i>slgC413</i>	NP187	
	NP436	<i>slgC423</i>	NP187	
	NP438	<i>slgC425</i>	NP187	
<i>slgD</i>	KY3	<i>slgD700</i>	NC4	*NP389 (<i>tsg-400</i>)
<i>slgE</i>	*NP392	<i>slgE404</i>	XP55	XP183 (<i>tsgA1 bsgA⁺</i>)
	NP422	<i>slgE410</i>	NP187	XP267 (<i>bsgB500 tsgK⁺</i>)
	NP429	<i>slgE416</i>	NP187	
<i>slgF</i>	NP428	<i>slgF415</i>	NP187	
	NP430	<i>slgF417</i>	NP187	
	NP440	<i>slgF427</i>	NP187	
	NP441	<i>slgF428</i>	NP187	
	NP448	<i>slgF435</i>	NP187	
	NP452	<i>slgF439</i>	NP187	*XP228 (<i>bsgB500 tsgK⁺</i>)
	NP453	<i>slgF440</i>	NP187	XP243 (<i>bsgB500 tsgK⁺</i>)
<i>slgG</i>	NP409	<i>slgG407</i>	XP55	NP418 (<i>tsgK21 bsgA⁺</i> reverted)
	*NP410	<i>slgG408</i>	XP55	
<i>slgH</i>	*NP449	<i>slgH436</i>	NP187	XP317 (<i>bsgC350 tsgK21</i>)
<i>slgI</i>	*NP435	<i>slgI422</i>	NP187	XP241 (<i>bsgA5 tsgK⁺</i>)
<i>slgJ</i>	*NP439	<i>slgJ426</i>	NP187	XP258 (<i>bsgA5 tsgK21</i>)

some instances the *slg* mutation was reverted (or suppressed) along with reversion (or suppression) of the *tsgK21* marker, and in one case the *slg* mutation was rendered temperature-sensitive (allowing sporulation at 22 °C but halting development at the slug stage above 26.5 °C). Consequently, use was made of the recently discovered *bsgC* mutation on linkage group IV (Welker & Williams, 1980*a*) to allow diploids to be selected between *bsgC* derivatives of *slgH* and *bsgA* derivatives of *slgJ* at 22 °C.

Linkage analysis of slugger loci

Tester strains. Because the slugger mutations were introduced into several different parental strains, variously marked tester strains were required for linkage analysis. As this study was conducted over a period of several years, some of the early experiments with mutants of groups *slgA*, *slgB* and *slgC* used tester strains such as XP57, XP64 and XP86, which were later superseded. In these cases, linkage analysis was repeated with the more

Table 3. *Pattern of phenotypes of haploid segregants selected from diploids heterozygous for the slugger mutations in each of the complementation groups*

By convention, the recombination segregants lie on the lower left, upper right diagonal in each set of four figures so that zeros on these diagonals indicate linkage. The zeros in parentheses result from the selection of the *cyc4* or *acr4* alleles by the haploid selection procedure. The haploid selective agents used were cycloheximide (CYC), methanol (METH) or benlate (BEN).

Parental diploid	Parental haploids	Slugger mutation	Slg/Slg ⁺ phenotype	Linkage group										Haploid selective agent		
				I	II		III		IV		VI		VII			
DP1115	NP451 XP232	<i>slgA438</i>	Slg Slg ⁺	Cyc	Cyc ⁺	Acr ⁺	Acr	Bsg ⁺	Bsg	Bwn ⁺	Bwn	Man ⁺	Man	Tsg	Tsg ⁺	METH
				19	22	10	41	41	0	26	15	41	0	21	20	
				15	25	10	40	0	40	26	14	0	40	11	29	
DP852	NP347 XP95	<i>slgB401</i>	Slg Slg ⁺	Cyc	Cyc ⁺	Acr ⁺	Acr	Bsg	Bsg ⁺	Bwn ⁺	Bwn	Man ⁺	Man	Cob ⁺	Cob	CYC + METH
				45	10	10	45	45	0	42	3	43	2	20	25	
				74	10	10	74	0	74	58	16	10	64	43	29	
DP849 DP850	NP351 XP76	<i>slgC402</i>	Slg Slg ⁺	Cyc ⁺	Cyc	Acr	Acr ⁺	Tsg ⁺	Tsg*	Bwn ⁺	Bwn	Man ⁺	Man	Cob ⁺	Cob	METH
				62	28	90	10	8	7	90	0	52	38	22	68	
				9	45	54	10	12	3	0	54	45	9	5	49	
DP934 DP935	NP389 XP144	<i>slgD700</i>	Slg Slg ⁺	Cyc ⁺	Cyc	Acr ⁺	Acr	Bsg ⁺	Bsg	Bwn ⁺	Bwn	Man ⁺	Man	Cob ⁺	Cob	METH
				5	48	10	53	53	0	34	19	32	21	36	17	
				6	22	10	28	0	28	23	5	14	14	17	11	
DP975 DP976	NP392 XP95	<i>slgE404</i>	Slg Slg ⁺	Cyc	Cyc ⁺	Acr ⁺	Acr	Bsg	Bsg ⁺	Bwn ⁺	Bwn	Man ⁺	Man	Cob ⁺	Cob	CYC
				43	10	43	0	28	15	29	14	28	15	17	26	
				76	10	0	76	59	17	46	30	59	17	45	31	

DP1111	NP441 XP232	<i>slgF428</i>	Slg	Cyc	Cyc ⁺	Acr ⁺	Acr	Bsg ⁺	Bsg	Bwn ⁺	Bwn	Man ⁺	Man	Tsg	Tsg ⁺	CYC, METH
			Slg ⁺	71	23	21	73	94	0	76	18	94	0	25	69	
				2	17	0	19	0	19	18	1	0	19	8	11	
DP996 DP997	NP409 XP95	<i>slgG407</i>	Slg	Cyc	Cyc ⁺	Acr ⁺	Acr	Bsg ⁺	Bsg ⁺	Bwn ⁺	Bwn	Man ⁺	Man	Cob ⁺	Cob	METH
			Slg ⁺	35	170	[0]	205	205	0	43	162	203	2	128	77	
				5	13	[0]	18	0	18	8	10	0	18	9	9	
DP1100	NP449 XP232	<i>slgH436</i>	Slg	Cyc	Cyc ⁺	Acr ⁺	Acr	Bsg ⁺	Bsg	Bwn ⁺	Bwn	Man ⁺	Man	Tsg	Tsg ⁺	CYC, METH
			Slg ⁺	10	7	8	9	17	0	11	6	15	2	17	0	
				49	26	24	51	65	10	51	24	57	18	0†	75	
DP1099	NP435 XP232	<i>slgI422</i>	Slg	Cyc	Cyc ⁺	Acr ⁺	Acr	Bsg ⁺	Bsg	Bwn ⁺	Bwn	Man ⁺	Man	Tsg	Tsg ⁺	METH
			Slg ⁺	4	17	[0]	21	19	2	21	0	18	2‡	4	17	
				60	36	[0]	96	82	14	0	96	68	16	12	84	
DP1120	NP439 XP232	<i>slgJ426</i>	Slg	Cyc	Cyc ⁺	Acr ⁺	Acr	Bsg ⁺	Bsg	Bwn ⁺	Bwn	Man ⁺	Man	Tsg	Tsg ⁺	BEN
			Slg ⁺	19	15	6	28	19	15	29	5	12	11‡	34	0	
				20	51	9	62	51	20	48	23	36	18	0	71	

* Because of the presence of *tsgD12* linked to *acrA*, the Tsg/Tsg⁺ phenotype could not be determined in haploids selected on plates containing methanol. These figures were taken from an independent haploidization of the same diploids using cobaltous chloride selection and scoring the Cyc Acr⁺ segregants.

† Segregants in this class have been observed in other experiments by us and by J. S. Wallace (unpublished); the zero in this experiment arose because all the Tsg segregants happened to be Bsg⁺ (see text).

‡ In these cases not every segregant was tested for its Man phenotype after it was clearly established that both Man and Man⁺ non-parental phenotypes were represented.

Table 4. Assignment of complementation groups *slgA*–*slgJ* to linkage groups, based on the pattern of segregation shown in Table 3

The distinction between linkage groups III and VI in the assignment of *slgA*, *slgF* and *slgG* is ambiguous (possibly due to translocation). The *SlgH* phenotype requires both linkage groups III and VII from the mutant strain as described in the text.

Complementation group	Linkage group
<i>slgA</i>	III/VI
<i>slgB</i>	III
<i>slgC</i>	IV
<i>slgD</i>	III
<i>slgE</i>	II
<i>slgF</i>	III/VI
<i>slgG</i>	III/VI
<i>slgH</i>	III + VII
<i>slgI</i>	IV
<i>slgJ</i>	VII

recently constructed tester strains that allowed simpler interpretation of the phenotypic pattern; for brevity, only the latter results are shown in Table 3. Derivatives of parental strain XP55 were fused with tester strain XP95 which has been described previously (Ratner & Newell, 1978). Tsg derivatives of the slugger mutants obtained from M. Sussman were fused with either XP76 or XP144. Derivatives of parental strain NP187 were fused with XP232 which was specifically constructed as the appropriate tester for this strain.

Segregation of diploids. In most cases, haploidization of the diploids constructed between the slugger mutants and the tester strains was achieved by selection for the *acrA* marker using agar plates containing 2% (v/v) methanol. In cases where such selection caused problems, such as for *slgE* which is linked to *acrA*⁺ on linkage group II, the *cycA* selector was used with plates containing 500 µg cycloheximide ml⁻¹. In the later phase of the study the haploidizing agent benlate was also employed at 20 µg ml⁻¹ (Williams & Barrand, 1978). Although this agent generally gave good segregation patterns for most linkage groups, it showed a considerable bias for selection of the *acrA* marker on linkage group II due to cross-resistance of this marker for benlate resistance (see results for *slgJ*, Table 3).

Complexities of the phenotypic pattern. For most of the data in Table 3, the pattern of phenotypes allows unambiguous assignment of the linkage groups of the slugger mutants (Table 4). However, in certain cases the pattern is reproducibly complex. For example:

(a) Due to co-segregation of linkage groups III and VI in experiments employing *slgA*, *slgF* and *slgG* mutants, the phenotypic pattern of segregants cannot distinguish in which of these two linkage groups the mutations are located. Such co-segregation has been noticed in previous studies with related strains (Ross & Newell, 1979; Welker & Williams, 1980*b*) and may be due to a translocation in the strains used. The effect is seen most strongly with the parental strain NP187 but usually to a lesser degree with strain XP55, as may be noticed with some of the XP55-derived slugger mutants in Table 3. In the case of *slgF*, all seven mutants isolated were in the NP187 background and showed absolute co-segregation of linkage groups III and VI. In the case of *slgA*, six of the seven mutants isolated were also in this strain and produced similar results. The seventh *slgA*-bearing strain was isolated in an XP55 background. Linkage analysis of this strain again showed nearly complete co-segregation of linkage groups III and VI. In 153 segregants tested with this strain only one exception was found to the III/VI co-segregation pattern and (after rigorous examination of its phenotype) this indicated that *slgA* is linked to group VI rather than group III. In the case of *slgG*, the data from two out of 223 segregants indicated that this mutation is located on linkage group III rather than VI. However, in view of the relatively small numbers of segregants involved these assignments must be stated tentatively.

(b) Strong bias may at times be noticed in the phenotypic pattern. However, as long as one or two segregants of each of the four phenotypic classes can be rigorously established for any particular linkage group, then the bias may not influence the interpretation of the linkage assignment. In some cases, however, the complete absence of a class of segregant could be interpreted as evidence for the involvement of more than one linkage group for the slugger phenotype, or the presence of slugger suppressor mutations in the tester strain being used. An example of this type of effect was obtained with haploidization of diploid DP1105, constructed from the slugger strain NP433 (derived from NP187) and tester strain XP232. The pattern of phenotypes produced by this diploid strain with cycloheximide or methanol selection showed no clear linkage to any particular linkage group but three classes of segregant on linkage group IV. The failure to find only two classes of segregant for any of the marked linkage groups suggests that the *slg* mutation being studied is on linkage group V (which is as yet unmarked). However, the appearance of only three classes of segregant on linkage group IV (with an absence of the slugger non-parental class) also suggests that the parental linkage group IV from the slugger strain may be essential for the slugger phenotype. This makes the analysis of this particular pattern unresolvable with our present lack of understanding of linkage group V, and this mutant has not been included in the data presented in Tables 2 and 3 of this study. A basically similar, but more analysable result, was found with *slgH*. In this case, three classes of phenotypes were found for linkage groups III and VII, the missing classes being Slg Bsg on III and Slg Tsg⁺ on VII (see Table 3). This finding suggests that both linkage groups III and VII of the slugger strain are required for expression of the slugger phenotype, possession of only one of these linkage groups giving a phenotype that is wild-type. In principle, such complex linkage results may cause misinterpretation of complementation data. In the case of *slgH436*, the data showing *slgH* as a separate complementation group has been retained, as the only other slugger showing any requirement for linkage group VII (*slgJ*) did not show such effects and was clearly phenotypically distinct from the *slgH* mutation. The status of *slgH* remains tentative, however, as the possibility cannot presently be ruled out that a mutation on linkage group III or VII which gives only a partial effect in *slgH* is able to produce a complete slugger phenotype in other strains.

Sometimes an apparent bias and missing phenotypic classes may be seen only with one particular selector. In the case of *slgF* there was a strong bias for the Slg class of segregants when using selection of haploids with cycloheximide, which in some segregations led to missing classes of segregant. Fortunately, with selection on methanol plates this bias was not apparent, and both Slg Cyc and Slg Cyc⁺ classes freely segregated. The data shown in Table 3 for *slgF* are the combined data from an experiment that employed both methanol and cycloheximide selection plates (independently) so that the bias due to the cycloheximide (which was absolute in this experiment) appears as a single zero in the Slg⁺Acr⁺ class. Because of the results found with methanol, any explanation of the cycloheximide bias in terms of the need for linkage group I (bearing *cycA5*) of the parental strain would seem to be ruled out.

Phenotypes of complementation groups A to J

Examination of the phenotypes of the mutants developing on filters in the dark revealed significant and consistent differences between the groups. The phenotypes are shown diagrammatically in Fig. 1. The normal wild-type pattern of development passes through the finger and slug stage for a period that lasts approximately 5 h under the standard conditions used. In the mutants this period is extended for many hours, in some cases indefinitely. With *slgA* mutants a normal migrating slug stage is formed which migrates for about 60 h before taking on a withered appearance as the slug ages and the cells eventually die. With *slgB* the mutants again migrate for long periods and characteristically leave behind a slime trail that appears deformed with a somewhat crystalline appearance. Mutants of *slgC* form very large

Fig. 1. Schematic representation of the morphology of the developmental stages of the wild-type strain and mutants of complementation groups *slgA*–*slgJ*. For all except *slgF* the timing is that seen at 22 °C in the dark, the cells being incubated at a density of $5 \times 10^6 \text{ cm}^{-2}$ on 47 mm diameter Whatman no. 29 filter discs (cut into quarters) supported by Whatman no. 17 pads saturated with LPS containing 40 mM-phosphate buffer as described by Newell *et al.* (1969). Under these conditions *slgF* cells aggregate poorly and so the timing shown is that seen in clones growing on SM agar inoculated with *K. aerogenes*. Note that for most groups the morphology and duration of the stages is influenced to some extent by the cell population density. This effect is particularly noticeable for *slgD* and *slgE* which, under conditions of higher density on SM agar, can form greatly extended aerial slime trails. The bead-like structures formed on the trails of slugs of *slgF* mutants are drops of liquid that do not contain spores.

The strains used as representatives of the different *slg* complementation groups were: *slgA*, NP208; *slgB*, NP347 and NP388; *slgC*, NP351 and NP396; *slgD*, NP389; *slgE*, NP392; *slgF*, NP452 and NP453; *slgG*, NP409 and NP410; *slgH*, NP449; *slgI*, NP435; *slgJ*, NP439.

slugs that migrate normally for up to 60 h. Two representatives of this complementation group (JC2 and JC4) have been examined in detail by Sussman *et al.* (1978) and found to synthesize the developmental enzymes (UDPglucose pyrophosphorylase, UDPgalactose epimerase and UDPgalactose transferase) that would be expected for the wild-type strain induced to form an extended slug migration phase. We confirmed the finding of Sussman *et al.* (1978) that the defect in *slgC* mutants was not principally an over-sensitivity to the slug migration stimulus (ammonia) but probably the failure to produce normal amounts of the fruit-inducing stimulus (fruit juice), since these mutants were induced to form fruiting bodies by addition of sodium propionate to the medium, as described by Sussman *et al.* (1978). The phenotype of *slgD* [only one member of which (KY3) has been observed so far] has been detailed previously by Yanagisawa *et al.* (1967). The phenotype of KY3 is highly dependent on the cell density: at low cell density ($5 \times 10^5 \text{ cm}^{-2}$) it forms fruiting bodies with almost normal appearance in the normal time (although with only prespores being formed in the spore masses), but at high cell density ($1 \times 10^7 \text{ cm}^{-2}$) it forms slugs which migrate for an extended period, eventually producing aerial 'slugs' supported by stalk-like slime trails. By switching staled substrata from the wild-type and the mutant, it has been established that KY3 is over-sensitive to the slug-forming stimulus (ammonia) (Newell & Sussman, cited in Newell, 1971; Schindler & Sussman, 1979) and this would explain the density dependence of the phenotype first noted by Yanagisawa *et al.* (1967). Mutants of *slgE* are phenotypically very similar to mutant KY3 and show a similar density dependence and formation of aerial slugs. Using the technique of ammonia removal by the enzyme NAD^+ -linked L-glutamate dehydrogenase with NADH and 2-oxoglutarate in the substratum or sprayed on to the slugs, as described by Schindler & Sussman (1977), *slgE* mutant NP392 was also found to resemble KY3 in forming fruits under conditions in which ammonia was not allowed to accumulate. Genetically, however, these two mutants are clearly distinct, KY3 (*slgD*) being on linkage group III and complementing NP392 (*slgE*) on linkage group II. Because of problems with poor aggregation of mutants of *slgF* on filters, the phenotype shown in Fig. 1 is that seen on SM agar. The *slgF* mutants form trails that lift the slug off the substratum and which with age can collect droplets of fluid along their length. Members of *slgG* differ from the other sluggers in only reaching the upright slug (or 'finger') stage and can rarely be induced to migrate. They are also temperature-sensitive such that the fingers tend to collapse into mounds when the temperature is raised above 23–24 °C. Although on SM agar *slgG* mutants do not progress beyond the finger stage, on filters the phenotype is clearly population-density dependent and at decreasing densities an increasing proportion of fingers are able to go on to form fruiting bodies. This formation is accelerated by the ammonia-removal enzyme system and by suspending upper pads saturated with 0.5 M- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 6.0) above the developing fingers. The *slgH* phenotype is distinctive in forming a fat migrating slug that shows no tendency to form fruiting bodies at any population density, while *slgI* tends to form blunt-nosed slugs that often migrate in a leaning position rather than horizontal to the substratum. The *slgJ* phenotype is distinctive in forming a rosette of tiny slugs that branch out from each original aggregate. These are capable of migrating but generally do not move very far on SM agar plates.

Comparative experiments with the ammonia-removal enzyme system indicated that only *slgD*, *slgE* and *slgG* are affected by removal of ammonia and hence over-sensitive to this metabolite, while addition of sodium propionate (the agent that mimics fruit juice) to all groups indicated that only *slgC* is induced to fruit by the presence of this chemical.

A general property of the slugger mutants observed is that although the genetic background can strongly influence the phenotypic appearance, the phenotypes of mutants in the same complementation group isolated in the lightly marked parental strains used in this study are very similar. The mutants may often show differences in the size of the slugs formed (this being influenced by factors affecting the territory size) but the characteristics of the particular complementation group are generally still apparent.

DISCUSSION

Complementation tests with 34 slugger mutants established 10 complementation groups. As four of these groups contain only one member it would seem likely that not all slugger genes are represented in this study. Assuming that all loci involved are equally mutable, statistical calculations from the complementation data indicate that the probability of there being more than 10 loci is significant ($P = 0.38$) but the probability of there being more than 12 is relatively small ($P = 0.03$).

The pattern of loci found from linkage analysis shows that the *slg* loci are scattered over four of the seven linkage groups without obvious evidence for gene clustering (although more groups may be associated with linkage group III than with any of the other linkage groups). Such scattering of genes associated with a particular phenotype is probably the normal pattern for this organism as a similar lack of clustering has now been reported for 10 'aggregateless' (*agg*) loci (Williams & Newell, 1976; Coukell, 1975, 1977), 10 radiation sensitivity (*rad*) loci (Welker & Deering, 1976, 1978; Coukell & Cameron, 1979) and six streamer (*stm*) loci (Ross & Newell, 1979, 1981).

During the linkage analysis, complexities were found in the pattern of segregation of some of the markers. Some of these have been discussed previously (Newell, 1978; Ross & Newell, 1979; Welker & Williams, 1980*b*). However, one such problem, concerned with the requirement for more than one linkage group for the phenotypic pattern, was not previously encountered in so clear a manner. The finding that mutant NP449 (*slgH*) required both linkage groups VII and III of the slugger strain in order to show the slugger phenotype, may be an example of an interaction of structural or (more probably) regulatory genes, both of which must be mutated for production of the observed aberration. It seems probable that this type of effect is more common than generally stated as many developmental mutations are 'affected' to some extent by the genetic background into which they are placed. Whether the effect of one of the linkage groups that seem to be required is strictly due to a mutation within that linkage group (an 'enhancer gene', for example) or is due to a suppressor gene located in the same linkage group in the tester strain that was used to construct the diploids is not always easily resolvable and may be a question of semantics if the suppressor is found to be widely distributed.

The main purpose of this study was to identify the genetic loci involved with the slugger phenotype, and the nature of the aberrations associated with the mutations was only briefly considered. Because the formation of the slug stage is a behavioural response to a crowded environment that is monitored by ammonia production (formed by breakdown of cellular protein for energy under starvation conditions), any defect leading to over-sensitivity in the monitoring system would lead to excessive stimuli for slug formation. Three of the slugger groups (*slgD*, *slgE* and *slgG*) show such over-sensitivity and can be induced to fruit by efficient removal of ammonia. The effect of sodium propionate, which was found by Sussman *et al.* (1978) to stimulate fruiting body formation in the mutants JC2 and JC4, was confirmed and another mutant (NP351) of the same complementation group (*slgC*) was tested and found to behave in a similar way. Presumably the mutants of this complementation group have a block in the formation of the natural fruit-inducing compound. Another metabolic defect that would be expected to lead to the slugger phenotype would be the over-production of ammonia, giving a dominant slugger phenotype in synergistic mixtures with the wild-type, but so far this class of mutant has not been identified.

The slugger mutants present the opportunity to explore the regulation of a behavioural response at a very simple level. Some, at least, of the defects should be readily resolvable while others may be far more deeply involved with the regulation of development than current thinking will elucidate. In this connection it is interesting that a study of these mutants by Harry MacWilliams of University of Massachusetts, Worcester, U.S.A. (personal communication) has revealed that while most of the mutants show normal ratios of prespore to

prestalk regions in the slugs (as demonstrated by vital staining with neutral red) the three members of the *slgE* group all show an abnormal pattern with the anterior (prestalk) region being very small compared with the other mutants or the wild-type.

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