NEW LOCI IN *DICTYOSTELIUM DISCOIDEUM* DETERMINING PIGMENT FORMATION AND GROWTH ON *BACILLUS SUBTILIS*

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ABSTRACT

Seventeen independently isolated pigmentless (white) mutations in *Dictyostelium discoideum* are all recessive and fall into three complementation groups identifying two new *whi* loci in addition to the previously characterized *wh*A locus. *whi*B and *whi*C map to linkage groups III and IV, respectively. In addition, it was discovered that our laboratory stock of NC4, the wild-type strain from which these mutants were derived, has spontaneously lost the ability to grow on *Bacillus subtilis*. This new mutation, *bsg*B500, maps to linkage group VII and is not allelic to *bsg*A. *bsg*B500 is the first spontaneously derived mutation in *D. discoideum* that can be used to select heterozygous diploids, and for the first time allows genetic analysis to be routinely performed on strains derived from an unmutagenized background.

PARASEXUAL genetic analysis in *Dictyostelium discoideum* routinely employs multiply marked tester strains for assignment of new mutations to linkage groups (for review, see NEWELL 1978). Two haploid strains fuse to form a diploid, which subsequently haploidizes by random chromosome loss to give a population of amebae containing reassorted chromosomes. New mutations can then be assigned to linkage groups by examining their segregation patterns relative to those of the previously mapped mutations of the tester strain. Ideally, the markers in such tester strains should be easily distinguished, visible mutations that can be scored rapidly and reliably. Unfortunately, although several classes of visible mutations exist, such as aggregateless, most interfere with the production of spores. Two notable exceptions that have received widespread use are the pigmentation mutations, *whi* (white) and *bwn* (brown).

Mature, wild-type sorocarps of *D. discoideum* contain an extracellular lemon-yellow pigment (RAPER 1935; STAPLES and GREGG 1967) that fails to accumulate in strains carrying the mutation *whi*A1, originally isolated by SUSSMAN and SUSSMAN (1963) and used to define linkage group II by KATZ and SUSSMAN (1972). To date, this has been the only *whi* mutation used in linkage analysis in *D. discoideum*.

This report describes the linkage assignment and complementation analysis of 17 independently isolated white mutants that define two new loci, and a

spontaneous mutation that results in the inability to grow on *Bacillus subtilis*, defining a new *bsg* locus. These new markers should prove useful in facilitating genetic analyses in an organism that is becoming increasingly important as a model developmental system (LOOMIS 1975). All three markers are considerably easier to score than previously existing mutations on the same linkage groups; furthermore, the new *bsg* mutant now provides an unmutagenized, nontemperature-sensitive background for the routine genetic analysis of new mutations. This should be particularly important for the study of temperature-sensitive developmental mutants.

MATERIALS AND METHODS

Strains: The genotypes of the strains used in this study are listed in Table 1. All of these strains derive ultimately from the wild-type strain NC4. HL100 is a derivative of NC4 that spontaneously lost the ability to grow on *Bacillus subtilis*. HL21 is a derivative of HL100 and is temperature sensitive for growth. White mutants were isolated following mutagenesis of strain HL21 with N-methyl-N'-nitro-N nitrosoguanidine, employing methods previously described (YANAGISAWA, LOOMIS and SUSSMAN 1967), which resulted in approximately 0.1% survival. The nomenclature is based on that of DEMEREC *et al.* (1966) (see also KESSIN, WILLIAMS and NEWELL 1974). Genotypes are given in italics, while phenotypes are represented by the locus symbol in roman type with an initial capital letter. Wild type is designated by the superscript +.

Genetic manipulations: Heterozygous diploids were selected from two haploid strains by complementation of nonallelic mutations that render the cells temperature sensitive for growth (LOOMIS 1969) or unable to grow on *Bacillus subtilis* (NEWELL *et al.* 1977). Linkage analysis was accomplished by screening haploid segregants obtained from these diploids by selecting for recessive drug-resistance markers. Diploids homozygous for such markers, presumably arising through mitotic recombination, were identified by phenotype and characteristic spore size and shape (SUSSMAN and SUSSMAN 1963) and were excluded from genetic analysis.

Screening haploid segregants: The phenotypes of haploid segregants were screened using standard methods (WILLIAMS, KESSIN and NEWELL 1974; RATNER and NEWELL 1978). The exception was screening for brown segregants. We have found that shaking approximately 10^7 amebae in test tubes with 1 ml sterile BONNER'S solution (BONNER 1947) containing 400 μg/ml dihydrostreptomycin sulfate results in the appearance of the reddish-brown pigment within 36 hr. This is 1 week sooner than on plates and has the further advantage of avoiding cross-staining of adjacent colonies due to the solubility of the pigment. Brown can be scored independently of white.

RESULTS

Isolation of white mutants: 6,919 independently isolated mutagenized clones derived from HL21 were screened for lack of yellow pigmentation. Forty clones were selected initially, of which 17 were completely white and were used for further analysis.

Complementation tests: Diploids were formed between X9, which carries *whi*A1, and all 17 white mutants. Nine of the strains gave rise to diploids with white sorocarps and thus either carry new *whi*A alleles or dominant white mutations (Table 2). Eight of the strains gave rise to yellow sorocarps when crossed to X9 and therefore possess recessive white mutations not allelic to *whi*A. The mutation in HL33 was designated *whi*B500 and this strain was used for further complementation tests.

TABLE 1

Strains of D. discoideum

Strain	Parent	Genotype*												Reference†
		cyc	*acr*	*whi*	*axe*	*rad*	*bsg*	*nag*	*ebr*	*bwn*	*man*	*cob*	*tsg*	
HL21	HL100						B500						P500	1
HL51	HPS64/HL106	A1 or 5	A604		A1, B1	C44					A2		A1	2
HL52	HL33/HL204		A2	B500	A1		B500		404				B3	2
HL100	NC4						B500							1
HL106	HL101/XP99	A1 or 5	A1	A1			A5	A211			A2	A1	D12	3
HL204	HL106/NP167	A1 or 5	A2		A1		A5		404				B3	2
HPS64	HPS203/TS12		A604		A1, B1	C44							A1	4
NP167	NP12		A2		A1, B1				404				B3	5
X9	M28/NP14	A1	A1	A1						A1			D12	6
X36	NP62/NP68	A1			A1, B1					A1		A1	A1	7
XP99	XP64/XP71	A1 or 5					A5			A1	A2	A1		8

* Genotypic symbols are as follows: *cyc*, cycloheximide resistance; *acr*A, acriflavin and methanol resistance; *whi*, white sorocarp; *axe*, axenic growth; *rad*, radiation sensitivity; *bsg*, lack of growth on *B. subtilis*; *nag*, deficiency in N-acetylglucosaminidase; *ebr*, ethidium bromide resistance; *bwn*, brown pigment; *man*, deficiency in α-mannosidase; *cob*, cobalt resistance; *tsg*, temperature-sensitive growth.

† (1) The parent of HL21 was found to carry *bsg*B and was cloned and named HL100. *tsg*P500 maps to linkage group II, (MORRISSEY, unpublished); (2) this study; (3) MACLEOD and LOOMIS (1979); (4) WELKER and DEERING (1978); (5) isolated by K. L. WILLIAMS (unpublished) as cited by RATNER and NEWELL (1978); *ebr*-404 assigned to linkage group IV by P. C. NEWELL and K. L. WILLIAMS (personal communications); (6) KESSIN, WILLIAMS and NEWELL (1974); (7) WILLIAMS and NEWELL (1976); (8) RATNER and NEWELL (1978).

TABLE 2

Genotype and complementation data of* whi *strains*

Strain	allele	Sorocarp color when crossed to: X9	HL52
HL22†	*whi*A516	white	yellow
HL35	*whi*A503	white	yellow
HL36	*whi*A504	white	yellow
HL37	*whi*A505	white	yellow
HL39	*whi*A507	white	yellow
HL40	*whi*A508	white	yellow
HL41	*whi*A509	white	yellow
HL43	*whi*A510	white	yellow
HL45	*whi*A511	white	yellow
HL32	*whi*B501	yellow	white
HL33	*whi*B500	yellow	white
HL34	*whi*B502	yellow	white
HL38	*whi*B506	yellow	white
HL46	*whi*B512	yellow	white
HL47	*whi*B513	yellow	white
HL48	*whi*B514	yellow	white
HL49	*whiC*515	yellow	yellow

* In addition to the *whi* alleles, these strains also carry *bsg*B500 and *tsg*P500.
† This strain also carries an additional mutation, *stk*-503, which results in a stalk-only fruiting body, to be described elsewhere (MORRISSEY and LOOMIS, in preparation).

Since all of the mutants were isolated in the same genetic background, it was not possible to use HL33 directly for complementation analysis with the other white mutants. HL33 was crossed to HL204, from which a derivative carrying *acr*A2, *whi*B500, *ebr*-404, *tsg*B3 was selected (HL52). Selection for *acr*A2 eliminated *tsg*P500, which is in repulsion to it on linkage group II, while selection for *ebr*-404 brought in the linked marker, *tsg*B3.

HL52 was crossed to the other 16 mutants and the resulting diploids were scored for sorocarp color (Table 2). The nine white mutants that fail to complement *whi*A1 do complement *whi*B500; therefore, they are classified as recessive *whi*A alleles. Seven of the eight remaining mutants do not complement *whi*B500; these are then recessive alleles of *whi*B. The one exception, HL49, carries a recessive mutation in a third white locus, *whi*C.

Alhough these mutations were observed to complement in diploids, they appear to be cell-autonomous. This was determined by co-aggregating strains containing mutations at different *whi* loci in pairwise combinations, or by co-aggregating all three mutant classes. The resulting fruiting bodies were pigmentless, while coaggregation of white mutants with wild-type yielded yellow sorocarps (MORRISSEY, unpublished observations).

Linkage assignment of whi*B*: Strain HL33 was crossed to HL51 (to form DL3), from which haploid segregants resistant to methanol were selected. As

TABLE 3

Phenotypes of methanol-resistant segregants from DL3

	Linkage group								Linkage group	Haploid parents	
	I		III		VI		VII				
	Cyc+	Cyc	Tsg+	Tsg	Man+	Man	Bsg+	Bsg		HL33	HL51
Whi+	12	0	0	12	12	0	11	1	I		*cyc*A
Whi	100	3	103	0	102	1	80	23	II	*tsg*P	*acr*A *axe*A
									III	*whi*B	*axe*B *rad*C *tsg*A
Bsg+	88	3	80	11	90	1	—	—	VI		*man*A
Bsg	24	0	23	1	24	0	—	—	VII	*bsg*B	

Haploids were selected on 2% methanol, which selects for the recessive marker *acr*A. Since this selects against the chromosome bearing *tsg*P, the Tsg phenotype can result only from the possession of *tsg*A. The segregation data are presented twice—once with respect to Whi and again with respect to Bsg. Five diploids homoygous for *acr*A were excluded from these data.

shown in Table 3, the *whi*B500 marker segregated independently from markers on linkage groups I, II, VI and VII, while segregating in opposition to *tsg*A1, indicating that the mutation is on linkage group III. Thus, all the Whi$^+$ segregants were Tsg, and all the Whi segregants were Tsg$^+$. *tsg*A could be scored independently of *tsg*P, since selection of AcrA segregants selects against the chromosome carrying *tsg*P. This linkage assignment was confirmed by crossing HL33 to HPS64 and showing again that *whi*B500 segregated in opposition to *tsg*A1 (69 segregants were Whi Tsg$^+$; 31 were Whi$^+$ Tsg; none were Whi Tsg or Whi$^+$ Tsg$^+$). WELKER and WILLIAMS (personal communication) have confirmed that *whi*B500 is on linkage group III and segregates independently from markers on linkage group IV.

Two comments regarding these data must be made. The first involves the apparently nonrandom segregation pattern observed relative to many of the markers, such as the complete absence of the Whi$^+$ Cyc class of segregant from DL3. This phenomenon has been observed repeatedly in studies involving the parasexual genetics system in *D. discoideum*. It is thought to be caused by the possession by certain chromosomes of minor deleterious mutations that result in a biased segregation pattern owing to the growth interval between the spontaneous formation of haploids and their subsequent selection. Alternatively, skewing can arise from the effects of deleterious combinations of genes that are not themselves deleterious in the original genetic background. Skewing is discussed in detail by NEWELL (1978) and Ross and NEWELL (1979). The presence of yellow segregants possessing chromosomes derived from the HL33 parent (Cyc$^+$, Bsg, and Man$^+$ classes) unambiguously demonstrates lack of linkage.

The second point is that during the course of this study we noticed that none of the white strains nor their immediate parent, HL21, would grow on *B. subtilis*. The reason became clear when we found that our laboratory stock of NC4, from which all of these strains were derived, had spontaneously lost the ability to grow on this species of bacteria. Complementation analysis was carried out by crossing HL49 to XP99, which carries the *bsg*A5 mutation, and demonstrating

TABLE 4

Phenotypes of cycloheximide-resistant segregants from DL5

	Linkage group										Linkage group	Haploid parents	
	II		III & VII		IV		VI		VII			HL49	XP99
	Tsg+	Tsg	Bsg+	Bsg	Bwn+	Bwn	Man+	Man	Cob+	Cob			
Whi+	1	36	0	37	0	37	32	5	34	3	I		*cyc*A
Whi	4	32	4	32	36	0	33	3	31	5	II	*tsg*P	
											III		*bsg*A
Bsg+	1	3	—	—	4	0	2	2	0	4	IV	*whi*C	*bwn*A
Bsg	3	66	—	—	32	37	63	6	65	4	VI		*man*A
											VII	*bsgB*	*cob*A

Haploids were selected on cycloheximide (500 μg/ml), which selects for the recessive marker *cyc*A. The data are presented as in Table 3. Thirty-four diploids homozygous for *cyc*A were excluded from these data.

that the resulting diploid, DL5, would grow on *B. subtilis*. The mutation that our strains carry, *bsg*B500, defines a new *bsg* locus. Diploids between several *bsg*A5 and *bsg*B500 strains have been selected by growth on *B. subtilis* lawns at 22° (data not shown). The assignment of *bsgB* to linkage group VII is shown below.

Linkage assignment of whi*C:* whiC515 was assigned to linkage group IV, based on the segregation of DL5 presented in Table 4. This marker showed segregation in opposition to *bwn*A1, and independent segregation from markers on linkage groups I, II, VI and VII. This linkage assignment is consistent with the segregation data in Table 5 showing that *bwn*A1, but not *whi*C515, was recovered in segregants.

Linkage assignment of bsg*B:* *bsg*B500 was assigned to linkage group VII by crossing HL49 to X36 (to form strain DL4, see Table 5). Although the *whi*C515 marker was not recovered in this cross due to extreme skewing in favor of the *bwn*A1 chromosome, it can be seen that *bsg*B500 segregates in opposition to *cob*A1. D. L. WELKER and K. L. WILLIAMS (personal communication) have confirmed this linkage assignment and have also shown *bsg*B500 to be nonallelic to *bsg*C350, another newly defined *bsg* locus.

TABLE 5

Phenotypes of cycloheximide-resistant segregants from DL4

	Linkage group								Linkage group	Haploid parents	
	II & III		IV		IV		VII			HL49	X36
	Tsg+	Tsg	Whi+	Whi	Bwn+	Bwn	Cob+	Cob			
Bsg+	23	12	35	0	0	35	0	35	I		*cycA*
Bsg	7	20	27	0	0	27	27	0	II	*tsg*P	*axe*A
									III		*axe*B *tsg*A
									IV	*whi*C	*bwn*A
									VII	*bsg*B	*cob*A

Haploids were selected on cycloheximide (500 μg/ml), which selects for the recessive marker *cyc*A. Thirty-nine diploids homozygous for *cyc*A were excluded from these data.

Since *bsg*B500 arose spontaneously in a strain that was carried for a great many generations in association with *Klebsiella aerogenes*, it was necessary to eliminate the possibility that additional *bsg* mutations have accumulated on other linkage groups. From the data in Table 5 it may be seen that no additional *bsg* mutations can exist on linkage group III, since 23 segregants were Bsg^+ Tsg^+. This can occur only by receiving chromosome *II* from X36 and *III* from HL49. By a similar argument, it may be seen from the segregation pattern of DL3 (Table 3) that the occurrence of Bsg^+ Cyc^+, Bsg^+ Tsg^+, and Bsg^+ Man^+ segregants demonstrates the absence of *bsg* mutations on linkage groups I, III and VI, respectively. Table 4 shows data obtained from diploid DL5. Although this segregation is complicated by the presence of both *bsg*A5 and *bsg*B500, the classes that combine Bsg^+ with HL49-derived alleles are telling. Thus, the Bsg^+ Tsg, Bsg^+ Bwn^+, and Bsg^+ and Man^+ classes argue against additional mutations on linkage groups II, IV and VI, respectively, while the existence of Bsg^+ segregants confirms the absence of any *bsg* markers on III. The absence of the Bsg^+ Cob^+ class is consistent with the assignment of *bsg*B500 to linkage group VII.

Therefore, there are no additional *bsg* mutations on linkage groups I, II, III, IV or VI. Linkage group V is at present unmarked, but the presence of an independently segregating *bsg* mutation on this linkage group should have interfered with the scoring of *bsg*B *vs. cob*A. For example, in the cross presented in Table 5, some of the segregants would have received this additional unlinked *bsg* mutation, creating a Cob Bsg class, which was in fact not found.

DISCUSSION

The yellow sorocarp pigment in *D. discoideum* is known to be a carotenoid, probably a derived zeta-carotenoid (STAPLES and GREGG 1967). Synthesis of zeta-carotene involves a complex multistep pathway beginning with the condensation of farnesol pyrophosphate and isopentyl pyrophosphate (BONNER 1965). Since such a pathway may involve many gene products, it was of interest to know if additional *whi* loci exist.

Mutations in *whi*A and *whi*B were recovered at frequencies of 1.3×10^{-3} and 1.0×10^{-3}, respectively; the single mutation of the *whi*C locus gives a frequency of 1.4×10^{-4}. Chi-square analysis of the data indicates that the three *whi* loci are probably not equally mutable ($P < 0.05$), and that additional *whi* loci with apparent target sizes as large as those of *whi*A and *whi*B probably do not exist ($P < 0.02$).

The two new white loci should prove useful as genetic markers in further parasexual genetic analyses since they can be scored easily and reliably, while not interfering with morphogenesis or spore formation. They cannot, however, be used for the genetic analysis of developmental mutations such as those that block aggregation, since the yellow pigment is produced only by those strains that reach the culmination stage.

*bsg*B500 can be used as a general marker for linkage analysis, and in our hands is much more satisfactory than *cob*B, *stm*A or F (Ross and NEWELL 1979), which currently define linkage group VII. We have found the resistance to cobalt in *cob*A strains to be strongly affected by genetic background, and strains apparently Cob^+ show considerable variation in their ability to grow in the presence of cobaltous chloride (MORRISSEY, WHEELER and LOOMIS, unpublished observations).

In addition, *bsg*B500 is useful as a marker for the selection of diploids, either in conjunction with *tsg* mutations or with other *bsg* mutations. In the latter case, diploids can be selected on *B. subtilis* at 22° in the complete absence of *tsg* mutations. This should greatly facilitate analysis of mutations whose phenotypes are temperature sensitive. Since *bsg*B500 arose spontaneously in our wild-type stock, it will allow, for the first time, the isolation of new mutations in a completely unmutagenized background that is also amenable to routine genetic analysis.

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