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Genetic Characterization of Aggregation-Defective Developmental Mutants of *Myxococcus xanthus*

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The transposon Tn*5* was used to map temperature-sensitive mutants of *Myxococcus xanthus* defective in aggregation (C. E. Morrison and D. R. Zusman, J. Bacteriol. **140**:1036-1042, 1979). Seven of the eight mutants showing a similar terminal phenotype (rough) were found to be tightly linked. These mapped in a group of loci which we have designated *aggR1*, *aggR2*, *aggR3*, and *aggR4*. Temperature-sensitive mutants having a different terminal phenotype were not linked to *aggR*. A search through a group of nonconditional rough mutants indicated that a much lower proportion of these (1 of 35) mapped in *aggR*. Thus, *aggR* is probably only one of many sites which can lead to the rough phenotype when mutated. Localized mutagenesis was used to isolate nine additional *aggR* mutants. All mapped within *aggR1*, *aggR2*, or *aggR3*, and none was found outside this region. Thus, we have characterized a cluster of developmental genes which are needed for aggregation in *M. xanthus*. The localization of a Tn*5* insert adjacent to this region makes possible further manipulation of these genes.

Myxococcus xanthus is a gram-negative bacterium which exhibits cooperative behavior during its life cycle (9, 14). Under conditions of nutritional stress, cells cease vegetative growth and aggregate to form raised translucent mounds. Within these mounds of cells, individual rod-shaped bacteria convert to round or ovoid resting cells called myxospores. In *M. xanthus*, mounds of myxospores are termed fruiting bodies. The spores germinate when nutritional conditions improve.

To study particular steps within the developmental program, a large number of fruiting mutants were isolated which are blocked at specific stages of development (12). Some of these mutants are temperature sensitive, developing normally at 28°C but arresting at a characteristic terminal phenotype at 34°C. The mutants exhibit a variety of phenotypes, ranging from rough, in which groups of cells show some reassortment but very little aggregation, to translucent mounds, in which aggregation appears to proceed normally but sporulation is blocked. Intermediate phenotypes (swirl and flat mounds) are also found.

In an effort to understand the genetic complexity of aggregation and sporulation, we have begun to map a subclass of temperature-sensitive mutants that appear to arrest early in aggregation, although they generally sporulate normally (rough mutants). The mapping procedure used has been recently described (10) and involves the use of a bank of strains containing independent insertions of Tn*5*, a transposon imparting kanamycin resistance (Kan^r) (1), at multiple points in the chromosome. We located a strain which contains a Tn*5* insertion mapping near a rough nonfruiting mutant. The linkage of other mutants to this transposon was then assessed. This method has enabled us to determine that most of the temperature-sensitive rough mutants previously described (12) map within a group of tightly linked loci, which we have designated as *aggR1*, *aggR2*, *aggR3*, and *aggR4* (*agg*regation defective, *R*ough).

MATERIALS AND METHODS

Bacterial and bacteriophage strains and nomenclature. *M. xanthus* DZF1 (strain FB) was used as the parental wild-type fruiting competent strain (Fru$^+$). Temperature-sensitive Fru$^-$ mutants were previously described (12). The properties of these strains and others constructed in the course of this analysis are given in Table 1.

Pools of Tn*5*-containing *M. xanthus* strains were isolated after P1::Tn*5* infection of DK101 as described previously (10). The location of the Tn*5* transposons were named in terms of the strain in which they were first identified. Thus, DZF3080, the kanamycin-resistant derivative of strain DZF1589, contains the Tn*5* insert, Ω3080 (2).

Phage strains used in transductions were Mx8cp2, a clear-plaque variant of MX8 (10) and MX4ts27 *hft hrm* (3).

Media and growth conditions. Vegetative cultures were grown and maintained as described previously (12), except that CTT medium (6) was occasionally substituted for CYE. The morphology of fruiting

TABLE 1. *Description of strains of M. xanthus*

Strain designation	Relevant characteristics[a]	Fruiting phenotype at 34°C[b]	Source or reference
DK101	*sglA*	Fruiting	6
DZF1	*sglA*	Fruiting	12
DZF1098	Fru(Ts)	Translucent mound	12
DZF1207	Fru(Ts)	Swirl	12
DZF1224	Fru(Ts)	Rough	12
DZF1279	Fru(Ts)	Swirl	12
DZF1287	Fru(Ts)	Rough	12
DZF1442	Fru(Ts)	Flat mound	12
DZF1454	Fru(Ts)	Swirl	12
DZF1516	Fru(Ts)	Rough	12
DZF1552	Fru(Ts)	Rough	12
DZF1589	Fru(Ts)	Rough	12
DZF1601	Fru(Ts)	Rough	12
DZF1661	Fru(Ts)	Translucent mound	12
DZF1734	Fru(Ts)	Rough	12
DZF1760	Fru(Ts)	Translucent mound	12
DZF1963	Fru(Ts)	Translucent mound	12
DZF2873	Fru(Ts)	Rough	12
DZF3078	Tn*5* (Kan^r)	Fruiting	By transduction of DZF1552 with Mx8 grown on pools of DK101 containing Tn*5*
DZF3080	Tn*5* (Kan^r)	Fruiting	By transduction of DZF1589 with Mx8 grown on pools of DK101 containing Tn*5*
DZF3160	Tn*5* (Kan^r); Fru(Ts)	Rough	Localized mutagenesis of DZF1
DZF3161	Tn*5* (Kan^r); Fru(Ts)	Rough	Localized mutagenesis of DZF1
DZF3162	Tn*5* (Kan^r); Fru$^-$	Rough	Localized mutagenesis of DZF1
DZF3164	Tn*5* (Kan^r); Fru$^-$	Rough	Localized mutagenesis of DZF1
DZF3166	Tn*5* (Kan^r); Fru$^-$	Rough	Localized mutagenesis of DZF1
DZF3167	Tn*5* (Kan^r); Fru(Ts)	Rough	Localized mutagenesis of DZF1
DZF3168	Tn*5* (Kan^r); Fru$^-$	Rough	Localized mutagenesis of DZF1
DZF3169	Tn*5* (Kan^r); Fru(Ts)	Rough	Localized mutagenesis of DZF1
DZF3170	Tn*5* (Kan^r); Fru(Ts)	Rough	Localized mutagenesis of DZF1

[a] *sglA*, a locus involved in the S-motility system of *M. xanthus* (7); Fru(Ts), temperature sensitive for fruiting body formation; Fru$^-$, nonfruiting phenotype at 28 or 34°C.

[b] The fruiting phenotypes are described by Morrison and Zusman (12).

cultures was assessed by spotting samples of a concentrated cell suspension on CF agar as described previously (12).

Transducing phage. Mx8 lysates were prepared on donor strains and used in transduction as described previously (6, 11). Strains to be transduced to Kan^r were plated in a top agar overlay onto CF plates containing 15 μg of kanamycin sulfate (Sigma Chemical Co.) per ml. Plates were incubated at 34°C for about 10 days before scoring.

Since several Kan^r transductants isolated after phage Mx8 transduction appeared to be phage Mx8 resistant, most genetic analyses were performed with phage MX4. MX4 transductions were performed as described by Rudd and Zusman (13), except that phage stocks were prepared on plates containing 1% Trypticase (BBL Microbiology Systems), 0.1% $MgSO_4 \cdot 7 H_2O$, and 1.5% Difco agar. Stocks were harvested by flooding plates with 5 ml of TM buffer (10 mM Tris-hydrochloride, pH 7.6, containing 8 mM $MgSO_4$), incubating overnight at 4°C, and aspirating the supernatant. Phage stocks were clarified by centrifugation at 12,000 × *g* for 10 min and stored over chloroform.

Localized mutagenesis. Hydroxylamine mutagenesis of phage MX4 was performed essentially as described by Hong and Ames (8) for mutagenesis of phage P22. An MX4 phage stock was prepared on a Fru$^+$ Kan^r strain (DZF3080) and concentrated to 5 × 10^9 to 10 × 10^9 plaque-forming units per ml by centrifugation at 43,000 × *g* for 90 min. To 1 ml of phage suspension, we added 5 ml of 0.1 M $NaPO_4$ (pH 6) containing 1 mM EDTA and 4 ml of a freshly prepared solution of 1 M hydroxylamine adjusted to pH 6 with NaOH and containing 1 mM EDTA. The phage were incubated without shaking at 37°C for 24 to 48 h and harvested by centrifugation. This procedure resulted in a 50 to 90% decrease in phage titer. Mutagenized phage were used in transductions at an approximate multiplicity of 0.5 viable phage per recipient. Kan^r transductants of a Fru$^+$ strain (DZF1) were plated on CF agar containing kanamycin (15 μg/ml) and screened visually for Fru$^-$ clones after 10 to 14 days of incubation at 34°C. By this procedure, Fru$^-$ mutants linked to Ω3080 (the transposon present in the donor strain) were obtained at a frequency of 0.2%. In control experiments, in which 1,200 transductants arising after treatment with mock mutagenized phage were screened, five Fru$^-$ Kan^r strains were isolated, none of which was linked to Ω3080.

Stimulation of fruiting mutants by the wild

type. The ability of mutant strains to be stimulated by the wild type to form fruiting bodies was assessed as follows. Liquid cultures of Kan^r Fru(Ts) mutants (Ω3080 derivatives) and DZF1 (Kan^s Fru$^+$) were concentrated by centrifugation and suspended at about 7.5×10^9 cells per ml (about 1,500 Klett units, using a red filter) in cold TM buffer. Samples (10 μl) of mutant culture were spotted onto CF plates and allowed to dry. A 10-μl sample of concentrated DZF1 culture was then placed over the original spot. After a period of incubation at 34°C, kanamycin was added to some plates underneath the agar (final concentration of 50 μg/ml); others were incubated without the addition of kanamycin. Incubation was continued for 6 days, and fruiting morphology was scored. A positive response was indicated when a mixture of mutant and DZF1 showed a similar fruiting response.

RESULTS

Identification of a Tn*5* insertion which is linked to a temperature sensitive nonfruiting mutant. The morphology of fruiting body formation in *M. xanthus* is very simple, exhibiting but three distinct phases: (i) rough, the preaggregation stage; (ii) translucent mounds, the stage at which cells aggregate; and (iii) fruiting body formation, the stage at which the rod-shaped cells present in translucent mounds sporulate. Since the rough phenotype appears earliest, we decided to examine mutants blocked at this stage in more detail.

Kuner and Kaiser (10) have assembled a collection of *M. xanthus* strains containing independent insertions of the transposon Tn*5*. Pools of these strains, derived from 100 independent colonies, were used for growing stocks of the generalized transducing phage Mx8. The phage stocks were then used to infect several temperature-sensitive rough mutants previously characterized (12), and the cells were plated on CF agar containing kanamycin (15 μg/ml). On this medium, transductants to Kan^r give rise to colonies. If a strain is fruiting proficient (Fru$^+$), the colonies grow until the nutrients become limiting and then form fruiting bodies in situ. Thus, Fru$^+$ colonies are clearly distinguishable from Fru$^-$ colonies (Fig. 1).

When this procedure was performed with strain DZF1552, one Fru$^+$ Kan^r clone was identified after screening approximately 600 Fru$^-$ Kan^r colonies. This transductant (designated Ω3078) had presumably become Fru$^+$ through acquisition of a *fru*$^+$ allele linked to a Tn*5* carried by one of the original donor strains present in the pool. This was confirmed in the following way. The Fru$^+$ colony was purified on CTT plates containing kanamycin (20 μg/ml) and used as a donor for the preparation of an Mx8 phage stock. These phages were then used to transduce a fresh culture of DZF1552 to Kan^r

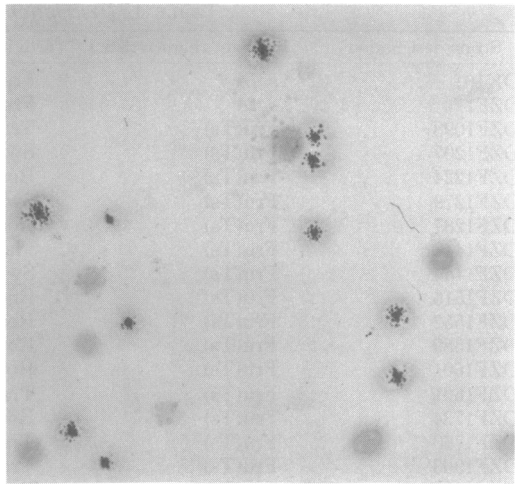

FIG. 1. *Colony morphology of fruiting and nonfruiting transductants on CF agar containing kanamycin (15 μg/ml). Fruiting clones appear as clusters of darkened fruiting bodies. Nonfruiting transductants appear as diffuse flattened colonies.*

and Fru$^+$. In this experiment, the insert Ω3078 was 67% linked to the temperature-sensitive fruiting [Fru(Ts)] mutation. Similar experiments allowed the identification of three other inserts near the Fru(Ts) mutation in strain DZF1589 (these were found at a frequency of 1/500). DZF1589, although initially described as a swirl mutant (12), appeared in our hands to show a temperature-sensitive rough phenotype. All four of the inserts described above were derived from the same pool of 100 donor Tn*5* containing strains and therefore may be identical. Subsequent experiments with two of these inserts, Ω3078 and Ω3080, gave consistently similar results (Table 2). Further experiments were therefore performed only with Ω3080.

Linkage of Ω3080 to other temperature-sensitive mutants. To determine whether any of the other Fru(Ts) strains previously described (12) were linked to the same Tn*5* insert (and consequently linked to each other), phage grown on strains DZF3080 and DZF3078 were used to transduce the remaining mutants to Kan^r. Of the Fru(Ts) rough mutants, seven of eight showed linkage frequencies very similar to those of the insert Ω3080 (Table 2). The remaining two rough mutants, DZF1308 and DZF1412 (which are not shown), may be double mutants, since a proportion of the Kan^r transductants isolated in such an experiment showed improved but not wild-type fruiting. In addition, phage grown on derivatives of DZF1308 and DZF1412 containing Ω3080 (and the *Fru* allele) could not transduce the wild-type parent, DZF1, to Fru$^-$. The re-

TABLE 2. *Linkage of Ω3080 and Ω3078 to temperature-sensitive fruiting mutants*[a]

Strain	Fruiting phenotype at 34°C	Frequency (%) of cotransduction of Fru$^+$ allele with:	
		Ω3080	Ω3078
DZF1516	Rough	65	68
DZF1552	Rough	54	66
DZF1589	Rough	47	59
DZF1287	Rough	43	58
DZF2873	Rough	46	49
DZF1224	Rough	41	37
DZF1734	Rough	43	42
DZF1601	Rough	<1	<1
DZF1454	Swirl	<1	<1
DZF1279	Swirl	<1	<1
DZF1207	Swirl	<1	<1
DZF1442	Flat mound	<1	<1
DZF1963	Translucent mound	<1	<1
DZF1098	Translucent mound	<1	<1
DZF1661	Translucent mound	<1	<1
DZF1760	Translucent mound	<1	<1

[a] Phage MX4 or Mx8 were grown on DZF3080 and DZF3078 and used to transduce the fruiting mutants to Kan^r. The mutants were plated on CF media containing 15 μg of kanamycin per ml. After 10 days of incubation at 34°C, the frequency of fruiting clones among all Kan^r colonies was determined by using a colony counter equipped with a magnifying glass. A minimum of 200 colonies were scored in each transduction. The frequencies given for Ω3080 represent the average of three separate experiments.

maining Fru(Ts) mutants described in Table 2, which are phenotypically distinguishable from the rough strains DZF1552 and DZF1589, also appear genetically unlinked to Ω3080.

Three-factor-cross analyses of mutants. To determine whether the Ω3080 linked rough strains were allelic, and if not, to determine their relative genetic positions, three-factor crosses were performed as illustrated in Fig. 2. Phage MX4 was grown on Fru(Ts) Ω3080 mutants and used to infect recipient Fru(Ts) strains. The cells were then plated on CF agar containing kanamycin, and the fraction of Kan^r Fru$^+$ tranductants was recorded after prolonged incubation at 34°C. To assure that the formation of these wild-type recombinants did not require four crossover events, the reciprocal transductions were also performed (Fig. 2). The results of these experiments are shown in Table 3. Wild-type recombinants arise from such crosses at a frequency above the reversion frequency (Table 3). The mutants are therefore not allelic. However, they appear to be tightly linked, as evidenced by the generally low frequency of wild-type recombinants. Table 3 allows an estimation of the recombinational distance between mutants, which is also shown in Fig. 3. Figure 3 expresses the distances between genetic markers as percent recombination between adjacent mutant sites. The difference between the recombination frequencies resulting from reciprocal crosses between adjacent mutants were averaged, since these values were thought to be more statistically valid than individual values. Crosses involving strain DZF1224 were not used for these calculations, however. Strain DZF1224 gives no recombinants when crossed with DZF1287 or DZF2873 and possibly one recombinant (out of 1,700 clones) with DZF1589. These results strongly suggest that DZF1224 contains a deletion or chromosomal rearrangement in this region or two closely linked mutations. The three-

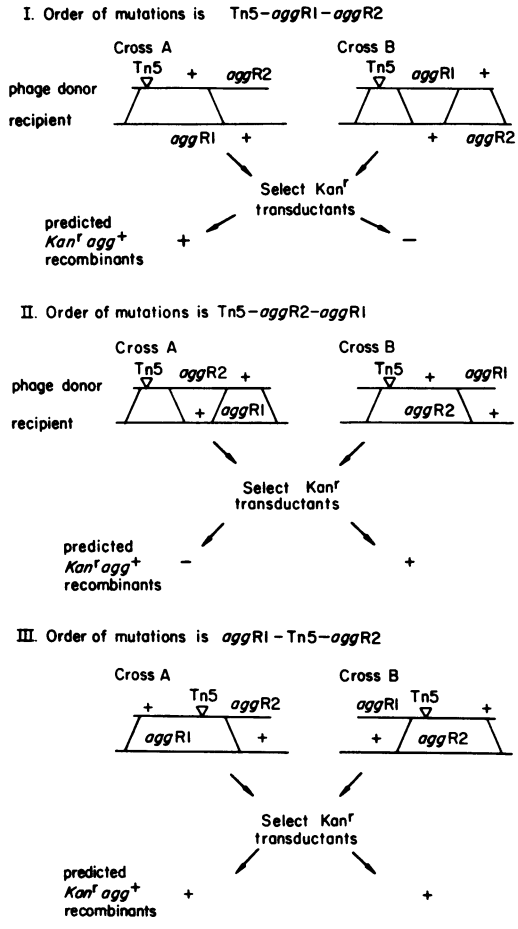

FIG. 2. *Predicted outcome of three-factor crosses of a stable insertion of Tn5 and two nonfruiting mutants (aggR1 and aggR2) based on three possible gene orders. The designations (+) or (−) are relative, implying only that recombinants requiring four crossover events are much less frequent than recombinants requiring two crossovers.*

point-cross data permit us to order the rough mutations, but without complementation tests, they do not permit us to assign the mutations to specific genes. However, since the recombination frequency between several of the loci is significant, we have tentatively assigned the mutations to four loci which we call *aggR1*, *aggR2*, *aggR3*, and *aggR4* (aggregation defective, rough mutants) (Fig. 3).

Are all rough mutants linked to Ω3080? Since the majority of the temperature-sensitive rough mutants tested were linked to Ω3080, we asked whether all rough mutants might be genetically linked. Thirty-five absolute (non-temperature sensitive) rough mutants were chosen at random from a collection of ICR- or ethyl methane sulfonate-mutagenized strains isolated by Morrison and Zusman (12) and screened for linkage to Ω3080 as described in Table 2. The majority of the mutants showed less than 1% linkage to Ω3080, with only one strain, DZF2798, showing 45% linkage. Although these mutants have not been carefully characterized to determine whether they contain one or more than one lesion, it is very likely that mutations in loci unlinked to Ω3080 can also result in a rough phenotype.

Isolation of absolute mutants linked to Ω3080 by localized mutagenesis. In an attempt to obtain additional mutations linked to Ω3080 which were not represented in the Fru(Ts) mutant collection, localized mutagenesis was used to isolate mutants linked to Ω3080. Phage MX4 was grown on strain DZF3080 (Fru$^+$ Kan^r) and then treated with hydroxylamine. The mutagenized phage were then used to transduce DZF1 (wild type) to Kan^r. Transductants were plated on CF agar containing kanamycin. Fru$^-$ Kan^r mutants were identified visually at an approximate frequency of 0.2%. These were single-

TABLE 3. *Three-factor crosses of rough fruiting mutants*

Recipient Fru(Ts) strains	Frequency (%) of Fru$^+$ recombinants among transductants carrying Ω3080[a] with donor Fru(Ts) Ω3080 strains:						
	DZF1516	DZF1552	DZF1287	DZF1589	DZF2873	DZF1224	DZF1734
DZF1516	**<0.02**	3.8	9.6	11	10.9	11.4	17.9
DZF1552	0.15	**<0.06**	2.9	2.9	3.8	3.5	5.5
DZF1287	0.21	0.15	**<0.1**	0.5	1.3	<0.03	3.3
DZF1589	ND	0.3	<0.1	**<0.03**	0.6	0.06	1.5
DZF2873	0.05	0.11	<0.3	<0.1	**<0.1**	<0.09	0.13
DZF1224	0.42	0.13	<0.06	0.1	<0.04	**<0.05**	<0.04
DZF1734	0.38	0.3	<0.8	0.5	<0.14	0.05	**<0.03**

[a] Phage MX4 was grown on DZF3080 and used to transduce the Fru$^-$ mutants listed above to Kan^r. Transductants which were Kan^r and Fru$^-$ were single-colony purified and used for the preparation of MX4 phage stocks. Transducing phage (which carry the donor DNA) were used to infect each of the recipient Fru$^-$ mutants and then plated on CF agar containing kanamycin (15 μg/ml). After 10 days of incubation at 34°C, the frequency of fruiting clones among the Kan^r transductants was determined. These numbers represent the average of 2 to 4 separate experiments in which a total of 700 to 7,500 transductants were analyzed. Mutants transduced with phage grown on strains containing the same allele gave no recombinants (boldface numerals). Data have been arranged so that recombinant classes requiring four crossover events appear below the boxes; productive crosses (requiring two crossover events) appear above them. ND, Not done.

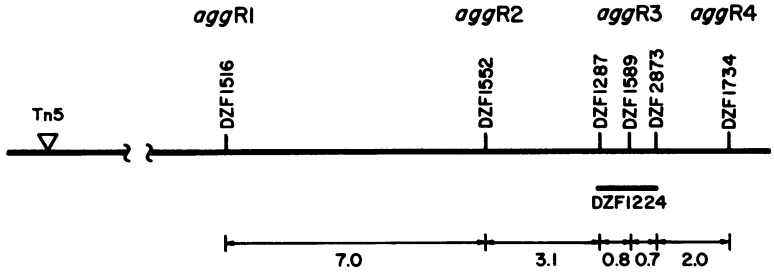

FIG. 3. *Genetic map of the aggR region of the M. xanthus chromosome. The two-point-cross data from Table 2 and the three-point-cross data from Table 3 were used to construct a genetic map of the Fru(Ts) mutants and the Ω3080 insert. The distances are expressed as percent recombination between adjacent mutant sites. The recombination frequencies were determined as described in the text. It should be noted that strain DZF1224 apparently contains a deletion or chromosomal rearrangement in the region covered by mutants DZF1287-DZF2873.*

colony purified on CYE plates containing kanamycin, and their morphology at 28 and 35°C was determined by spotting on CF agar (Table 4). Phenotypically, many of these mutants appeared rough. Several, however, had a darker, more granular appearance. These may represent leaky mutants. Many of the mutants were temperature sensitive (Table 4). The linkage of the mutations in these strains to Ω3080 was determined by cotransduction of the Fru⁻ phenotype with Kan^r. In these experiments, phage MX4 transducing lysates were grown on the mutants and then used to transduce DZF1 to Kan^r to determine the frequency of Fru⁻ Kan^r transductants. All of the mutants tested in this way were linked to Ω3080 (Table 4).

To determine the genetic relationship of these mutants to the temperature-sensitive rough mutants analyzed previously (Fig. 3 and Table 3), three-factor crosses were carried out. Since the mutants isolated by localized mutagenesis were already Kan^r, these crosses could not be performed in a reciprocal manner. MX4 phage were grown on the new mutants (which were Fru⁻ Kan^r) and used to transduce four reference strains (which were Fru⁻ Kan^s) to Kan^r. The number of Fru⁺ Kan^r recombinants were determined after 10 days at 34°C. The results of these crosses (Table 5) show that the mutants fall into three groups. One group (mutants DZF3160, DZF3168, and DZF3169) gave very few recombinants with any of the reference strains tested. These strains are either mutant in the *aggR1* locus or in another locus closer to Ω3080. However, these mutants gave approximately the same linkage frequencies to Ω3080 in two-factor crosses (Table 4) as did the *aggR1* mutant DZF1516 (63, 73, and 65% as compared with 65% for DZF1516); thus, they are probably *aggR1* mutants as well. Another group appears to be in *aggR2* and a third, in *aggR3*.

Phenotype of the Fru(Ts) rough mutants. Table 6 summarizes the fruiting phenotypes of the various *aggR* mutants previously described (12). In this study, we extended these findings to include the stimulation of fruiting by the wild type. Extracellular complementation (stimulation) of sporulation mutants was carefully investigated by Hagen et al. (5), who showed that about half of the nonsporulating mutants isolated could sporulate when mixed with the wild

TABLE 4. *Linkage of Ω3080 to fruiting mutants isolated by localized mutagenesis*[a]

Strain	Fruiting phenotype at:		Frequency (%) of cotransduction of Fru⁻ allele with Ω3080
	28°C	34°C	
DZF3162	Rough	Rough	48
DZF3164	Rough	Rough	41
DZF3168	Rough	Rough	73
DZF3170	Fruiting	Rough	56
DZF3160	Fruiting	Rough, granular	63
DZF3161	Fruiting	Rough, granular	53
DZF3166	Rough, granular	Rough, granular	63
DZF3167	Fruiting	Rough, granular	63
DZF3169	Rough, granular	Rough, granular	65

[a] Mutants isolated by localized mutagenesis were spotted onto CF agar and incubated at 28 or 34°C. The phenotypes of the mutants were determined after 5 days of incubation. Linkage of Ω3080 to the mutants was determined as described in the text. Approximately 200 Kan^r colonies were scored in each transduction.

TABLE 5. *Three-factor crosses of fruiting mutants isolated by localized mutagenesis with four reference strains*

Donor Fru⁻ Ω3080 strains[a]	Frequency (%) of Fru⁺ recombinants among transductants carrying Ω3080				Tentative locus assignment
	Recipient Fru⁻ strains				
	DZF1516 (*aggR1*)	DZF1552 (*aggR2*)	DZF1589 (*aggR3*)	DZF1734 (*aggR4*)	
DZF3160	0.4	<0.5	<0.6	<0.4	*aggR1*
DZF3169	0.9	<0.7	<0.7	<0.9	*aggR1*
DZF3168	1.0	<0.5	<0.5	<0.5	*aggR1*
DZF3167	1.5	<0.7	<0.5	<0.5	*aggR2*
DZF3166	3.6	<0.5	<0.8	<0.9	*aggR2*
DZF3161	5.1	1.6	<0.9	<1.1	*aggR3*
DZF3170	7.2	2.1	<0.8	<0.9	*aggR3*
DZF3162	11.7	3	<0.3	ND	*aggR3*
DZF3164	13.8	8	<1.3	<1.6	*aggR3*

[a] The fruiting mutants isolated by localized mutagenesis and containing Ω3080 were used for the preparation of MX4 phage stocks. The phage (donor) were then used to infect four nonfruiting strains analyzed in Table 3. The cells were plated on CF agar containing kanamycin (15 μg/ml). After 10 days of incubation at 34°C, the frequency of fruiting clones among the Kan^r transductants was determined. These numbers represent the average of two experiments; about 100 to 200 colonies were scored in each experiment. ND, Not done.

TABLE 6. *Summary of fruiting phenotypes of temperature-sensitive aggR mutants*

Strain	Tentative locus	Fruiting phenotype at 34°C[a]			Stimulation phenotype at 34°C[b]	
		Morphology	Sporulation	End of temperature-sensitive period (h)	− Kanamycin	+ Kanamycin
DZF1516	*aggR1*	Rough	+	9	Fruiting	Fruiting
DZF1552	*aggR2*	Rough	+	6	Fruiting	Fruiting
DZF1287	*aggR3*	Rough	±	12	Fruiting	Fruiting
DZF1589	*aggR3*	Rough	+	9	Fruiting	Fruiting
DZF2873	*aggR3*	Rough	−	15	Fruiting	Fruiting
DZF1224	*aggR3*	Rough	+	9	Rough	Rough
DZF1734	*aggR4*	Rough	+	9	Rough	Rough
DZF1	Wild type	Fruiting	+		Fruiting	Rough

[a] Data taken from Morrison and Zusman (12).

[b] The ability of mutants to fruit in the presence of the wild type was determined as described in the text. It should be noted that for these experiments, Kan^r derivatives of the mutants were used rather than the Kan^s strains listed above. The mutant phenotypes of these Kan^r strains, except for their resistance to kanamycin, were identical to those of the Kan^s strains.

type. Extracellular complementation was involved in their experiments, rather than genetic recombination, since analysis of clones derived from single spores showed the parental rather than recombinant phenotypes. Since most of the *aggR* mutants sporulate normally and are only defective in aggregation, we devised a new method (based on a suggestion by R. LaRossa) to examine whether wild-type cells could stimulate (complement) the mutants. Wild-type (Kan^s) and *aggR* mutants (Kan^r) were inoculated onto CF agar. After 3 h (long before any aggregation is observed in the wild type alone), kanamycin was added to the plates, blocking protein synthesis (and therefore aggregation) in the wild-type strain. However, strain mixtures (*aggR* mutants plus wild type) did aggregate and fruit in five of seven strains (Table 6). Two mutants did not fruit in the presence of the wild type (DZF1224 and DZF1734). In fact, these strains prevented the wild type from fruiting, even in the absence of kanamycin. These results suggest that most of the *aggR* mutants are capable of aggregation but fail to receive the signal to aggregate. The two mutants which inhibit wild-type aggregating may interfere with the signal in some manner.

DISCUSSION

We have used the transposon Tn*5* to map temperature-sensitive mutants of *M. xanthus* blocked in aggregation. Of eight mutants originally grouped together on the basis of their similar "rough" phenotype, seven appear genetically linked. All showed high linkage frequencies (65 to 40%) to Ω3080, the Tn*5* insert which was identified as being linked to one of the mutants. In contrast, mutants which showed different terminal phenotypes were not linked to these strains. Three-factor crosses were performed to determine the relative allele positions of the rough mutants and to determine the recombinational distance between the loci. On the basis of these crosses, we have separated the mutations into four groups, which we have tentatively designated *aggR1*, *aggR2*, *aggR3*, and *aggR4*, since significant recombinational distance separates the groups. Four mutants were placed in the *aggR3* group since these mutants appeared to be tightly linked (<1 recombination unit). However, it should be emphasized that none of the gene products corresponding to these loci have been identified, nor have complementation tests been performed. It therefore remains possible that the proposed *aggR* loci do not all correspond to separate genes or that, in contrast, they do not represent the only genes in this region. The size of the *aggR* genetic region can be estimated from the size of the generalized transducing phage used for these studies (molecular weight of about 39×10^6 [4]) and the frequencies of cotransduction between Tn*5*-Ω3080 and the proximal and distal markers, respectively (DZF1516, 65%, and DZF1734, 43%). If we assume a roughly proportional relationship between cotransduction frequency and DNA distance, then we can estimate the size of the DNA spanning *aggR1*-*aggR4* at about 8.6×10^6 daltons or about 1.3×10^4 nucleotide pairs. This should be more than enough DNA to accommodate the four proposed *aggR* genes.

The *aggR1*-to-*aggR4* region may represent the limits of the cluster of developmental genes linked to Ω3080 since nine of nine mutants isolated by localized mutagenesis map in this region. It is interesting that four of nine of these

mutants are temperature sensitive. There may be some special property to this region that permits these kinds of mutations at high frequency. This may explain why seven of eight nonsibling rough mutants in the original temperature-sensitive collection map in the *aggR1*-to-*aggR4* region, whereas only 1 of 35 rough non-temperature-sensitive mutants map in this region. Clearly, other genetic regions exist which give rise to absolute rather than temperature-sensitive mutants with the same rough morphology.

Comparison of genotype with phenotype (Table 6) in the original *aggR* mutants is easy to rationalize in the case of the *aggR1*, *aggR2*, and *aggR4* loci since they are each represented by only one mutant. In contrast, the four *aggR3* mutants appear to show somewhat differing phenotypes. This result may suggest that more than one gene is present in this region. An alternate hypothesis is that the gene products have more than one function and that they can be mutated independently.

In conclusion, we have used the transposon Tn*5* to locate a genetic region, *aggR*, which is involved in aggregation and fruiting body formation. Elucidation of the function of this genetic region awaits biochemical characterization of its products. The availability of absolute mutants and an adjacent selectable marker (Kan^r) should allow the use of molecular cloning for this purpose.

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