Genetic Analysis of *tag* Mutants of *Myxococcus xanthus* Provides Evidence for Two Developmental Aggregation Systems

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Temperature-dependent aggregation mutants (*tag*) of the myxobacterium *Myxococcus xanthus* aggregated into mounds and developed into fruiting bodies normally at 28°C; however, they failed to form mounds at 34°C. The timing of sporulation was unaffected by the mutations, and normal numbers of spores were produced at both permissive and restrictive temperatures. This class of mutations was originally identified through screening of ethyl methanesulfonate (EMS)-generated mutations. Subsequent work identified a linked insertion of transposon Tn5, which was used to map the EMS-generated mutations to four loci. In this paper, we describe the cloning of the *tag* loci and the use of transposon mutagenesis to further analyze the *tag* loci. Nine *tag* complementation groups spanning 8.5 kilobase pairs of DNA were identified through mapping of 28 independent Tn5 insertions. All insertion and deletion mutants had the same phenotype as the EMS mutants: they were temperature sensitive for mound formation. This result suggests that *M. xanthus* has at least two sets of genes for developmental aggregation. The *tag* genes constitute one set of these genes; they are required for normal development at 34°C but are not required for normal development at 28°C.

Myxococcus xanthus is a gram-negative myxobacterium found in a variety of soil habitats. It feeds upon other microorganisms and dissolved nutrients through the secretion of lytic and catabolic enzymes. Starvation triggers the developmental phase of the life cycle: most cells aggregate to form mounds which undergo morphogenesis into simple fruiting bodies. Five to 10% of the cells do not aggregate and remain as rod-shaped peripheral cells outside fruiting bodies. Within fruiting bodies, the rod-shaped cells differentiate into spherical spores. In the laboratory, the formation of fruiting bodies is morphologically complete within 48 to 72 h after the initiation of starvation. Cells within mounds begin to sporulate within 24 h of starvation. Maturation of spores continues for at least 6 days after spores first appear in the population (17, 22–25; unpublished data).

Our laboratory has been interested in the developmental program of *M. xanthus* (31). Morrison and Zusman (19) isolated a number of developmentally aberrant mutants through ethyl methanesulfonate (EMS) mutagenesis. Among these were mutants which were able to aggregate into mounds and develop normally at 28°C but which were unable to form mounds at 34°C. These mutants were described as “rough” because of the appearance of the surface of the mass of developing cells. Most of these mutants retained the ability to sporulate at the restrictive temperature. Torti and Zusman (28) used the method of Kuner and Kaiser (14) to identify a strain of *M. xanthus* which had a Tn5 insertion near one temperature-sensitive, rough mutation (i.e., the two loci were cotransducible). The linkage of other rough mutations to this Tn5 insertion (Ω 3080) was then assessed. Four loci were identified, and their relative order was established through two factor crosses. It was estimated that the loci would span 13 kilobase pairs (kbp) of DNA.

In this paper, we report the use of standard recombinant DNA techniques to clone the DNA coding for two of the four loci identified by Torti and Zusman (28). The transposition of Tn5 into the cloned DNA was used to create 28 new, independent mutations in the *M. xanthus* DNA. All of the

newly isolated mutants had the same phenotype as the original EMS-generated mutants (19) and the hydroxylamine-generated mutants (28): they were temperature sensitive for the ability to form mounds during development, but sporulation was unaffected (unpublished data). The ability of *M. xanthus* to fruit normally at 28°C in the absence of functional gene products in the Tn5 insertion mutants implies that these gene products are not required at 28°C but that they are required at 34°C. Therefore, the mutations are more properly referred to as temperature dependent rather than temperature sensitive. We have given them the designation *tag*.

P1 transduction (20) was used to perform complementation analyses of the *tag* mutations. Nine complementation groups (*tagA* to *tagI*), were identified; they are contiguous on the chromosome.

MATERIALS AND METHODS

Bacterial strains, media, and growth. Strains and bacteriophages are described in Table 1. *M. xanthus* was grown in Casitone-yeast extract (CYE) broth or in CYE broth containing 1.5% agar (5) at 30°C. Liquid cultures were shaken at 200 rpm. Fruiting body formation was assayed on clone fruiting medium containing 1.5% agar (CF agar) (8). *Escherichia coli* was grown in L broth or in L broth containing 1.5% agar (18). Antibiotics were used at the following concentrations: 100 mg of ampicillin per liter of L medium; 50 mg of kanamycin per liter of L medium or 30 mg of kanamycin per liter of CF agar; 10 mg of oxytetracycline per liter of CF agar or 12.5 mg of oxytetracycline per liter of CYE agar; and 25 mg of tetracycline per liter of L medium.

Materials. Ampicillin, CaCl₂, kanamycin sulfate, lysozyme, maltose, oxytetracycline, RNase A (type XII-A), and tetracycline were purchased from Sigma Chemical Co., St. Louis, Mo. Bacto-Agar, Casitone, tryptone, and yeast extract were purchased from Difco Laboratories, Detroit, Mich. All other chemicals (analytical-reagent grade) were from Mallinckrodt, Inc., St. Louis, Mo., purchased through Baxter Healthcare Corp., Scientific Products Div., McGaw Park, Ill. Pronase was purchased from CalBiochem Corp.,

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TABLE 1. Bacterial strains and phages^a

<i>M. xanthus</i> strain	Parent(s)	Mutagen	Tn5	Phenotype	Genotype	Comment	Reference(s) or source
DZF1		None	None	Kan ^s Tet ^s Fru ⁺	<i>tag</i> ⁺	<i>sglA</i> (Leaky)	7, 9
DZ2		None	None	Kan ^s Tet ^s Fru ⁺	<i>tag</i> ⁺	Wild type	7
DK1622	DK101	UV	None	Kan ^s Tet ^s Fru ⁺	<i>tag</i> ⁺	"Wild type"	12
DZF1516	DZF1	EMS	None	Kan ^s Tet ^s Tag ⁻	<i>tagC</i>	<i>aggR1</i>	28
DZF1552	DZF1	EMS	None	Kan ^s Tet ^s Tag ⁻	<i>tagE</i>	<i>aggR2</i>	28
DZF1589	DZF1	EMS	None	Kan ^s Tet ^s Tag ⁻	<i>tag?</i>	<i>aggR3</i>	28
DZF3080	DK2521 × DZF1589	NA	Ω3080	Kan ^r Tet ^s Fru ⁺	<i>tag</i> ⁺	Fru ⁺ transductant of DK2521 into DZF1589 as recipient	14, 28
DZF3144	DZF3080 × DZF1516	NA	Ω3080	Kan ^r Tet ^s Tag ⁻	<i>tagC</i>	<i>aggR1</i>	28
DZF3157	DZF3080	HA	Ω3080	Kan ^r Tet ^s Tag ⁻	<i>tagE</i>		28
DZF3159	DZF3080	HA	Ω3080	Kan ^r Tet ^s Tag ⁻	<i>tagC</i>		28
DZF3161	DZF3080	HA	Ω3080	Kan ^r Tet ^s Tag ⁻	<i>tagA</i>		28
DZF3163	DZF3080	HA	Ω3080	Kan ^r Tet ^s Tag ⁻	<i>tagE</i>		28
DZF3345	DZF3161	HA	Ω3080	Kan ^s Tet ^r Tag ⁻	<i>tagA</i>	Tet switch ^b	28
DZF3348	DZF3168	HA	Ω3080	Kan ^s Tet ^r Tag ⁻	<i>tagC</i>	Tet switch	28
DZF3414	DZF1	Tn5	Ω370 ^c	Kan ^r Tet ^s Tag ⁻	<i>tagC</i>	Gene replacement ^d	This paper
DZF3417	DZF1	Tn5	Ω374	Kan ^r Tet ^s Tag ⁻	<i>tagC</i>	Gene replacement	This paper
DZF3418	DZF1	Tn5	Ω376	Kan ^r Tet ^s Tag ⁻	<i>tagC</i>	Gene replacement	This paper
DZF3419	DZF1	Tn5	Ω379	Kan ^r Tet ^s Tag ⁻	<i>tagE</i>	Gene replacement	This paper
DZF3420	DZF1	Tn5	Ω381	Kan ^r Tet ^s Tag ⁻	<i>tagE</i>	Gene replacement	This paper
DZF3421	DZF1	Tn5	Ω382	Kan ^r Tet ^s Tag ⁻	<i>tagE</i>	Gene replacement	This paper
DZF3422	DZF1	Tn5	Ω383	Kan ^r Tet ^s Tag ⁻	<i>tagE</i>	Gene replacement	This paper
DZF3424	DZF1	Tn5	Ω385	Kan ^r Tet ^s Tag ⁻	<i>tagG</i>	Gene replacement	This paper
DZF3425	DZF1	Tn5	Ω386	Kan ^r Tet ^s Tag ⁻	<i>tagG</i>	Gene replacement	This paper
DZF3426	DZF1	Tn5	Ω387	Kan ^r Tet ^s Tag ⁻	<i>tagH</i>	Gene replacement	This paper
DZF3429	DZF1	Tn5	Ω367	Kan ^r Tet ^s Tag ⁻	<i>tag?</i>	Gene replacement	This paper
DZF3430	DZF1	Tn5	Ω378	Kan ^r Tet ^s Tag ⁻	<i>tagD</i>	Gene replacement	This paper
DZF3431	DZF1	Tn5	Ω368	Kan ^r Tet ^s Tag ⁻	<i>tag?</i>	Gene replacement	This paper
DZF3439	DZF1	Tn5	Ω369	Kan ^r Tet ^s Tag ⁻	<i>tag?</i>	Gene replacement	This paper

^a The *E. coli* strains used were C600 (*recA*⁺) (1) and HB101 (*recA*) (4). *recA*, Recombination system A. The phages used were P1 (host, *E. coli*) (29) and Mx4 (host, *M. xanthus*) (5). Abbreviations: *aggR1*, *aggR2*, and *aggR3*, loci necessary for aggregation of *M. xanthus* at elevated temperatures (19, 28); Fru⁺, wild-type fruiting phenotype in *M. xanthus*; HA, hydroxylamine; Kan^r, resistant to kanamycin at 75 µg/ml of CYE medium; Kan^s, sensitive to kanamycin; NA, not applicable; *sglA*, motility system S (9); Tag⁻, temperature dependent for aggregation; *tagA*, *tagC*, *tagD*, *tagE*, *tagF*, *tagG*, and *tagH*, loci identified in this study as necessary for aggregation of *M. xanthus* at elevated temperatures; Tet^r, resistant to oxytetracycline at 12.5 µg/ml; Tet^s, sensitive to oxytetracycline; Tn5, transposable element which confers resistance to kanamycin; Ω, Tn5 transposition, followed by the number of the strain in which it was obtained; ?, no *tag* locus assigned.

^b A strain containing a Tn5 insertion was infected with P1::Tn5-132, which confers tetracycline resistance. The transductants were selected for Tet^r and screened for Kan^s. All Kan^s Tet^r strains contained Tn5-132 at the site of the original Tn5 insertion; this result was confirmed by Southern blot hybridization analysis (6).

^c See Table 3 and Materials and Methods for a description of Tn5 insertions Ω361 to Ω388.

^d The gene containing the Tn5 insertion replaced the wild-type gene by homologous recombination; the strain was not merodiploid for the gene (20). The construction of these strains was confirmed by Southern blot hybridization analysis (6).

San Diego, Calif. All other enzymes, including restriction endonucleases, were purchased from Life Technologies, Inc., Gaithersburg, Md. Agarose was obtained from Bio-Rad Laboratories, Richmond, Calif. Nucleotides were purchased from Pharmacia LKB Biotechnologies, Inc., Piscataway, N.J. Radioactively labeled nucleotides were purchased from Amersham Corp., Arlington Heights, Ill. Nitrocellulose (0.45-µm pore size) was purchased from Sartorius, Hayward, Calif.

Cloning and subcloning *tag*. Chromosomal DNA was purified from DZF3080. Cells were grown in CYE to a concentration of 2×10^8 to 4×10^8 /ml, harvested by centrifugation at $12,000 \times g$ for 10 min at 34°C, and suspended in 50 mM Tris (pH 8.0) containing 1% sodium dodecyl sulfate. Pronase, preincubated at 37°C for 1 h, was added to a final concentration of 20 mg/liter; RNase A, preincubated at 100°C for 10 min, was added to a final concentration of 1 mg/liter. The mixture was incubated at 37°C for 3 h. The sample was extracted two times with buffer-saturated phenol, two times with buffer-saturated phenol-CHCl₃-isoamyl alcohol (25:24:1), and one time with CHCl₃-isoamyl alcohol (24:1). To the extracted preparation was added 2.5 volume

equivalents of 100% ethanol, and DNA was spooled out and suspended overnight in 10 mM Tris (pH 7.5)-1 mM EDTA (pH 7.5). DNA was quantitated from the A₂₆₀. Chromosomal DZF1 DNA was digested to completion with restriction endonuclease *EcoRI*, ligated into the *EcoRI* site of pHC79 (11) by standard recombinant DNA techniques, and packaged into lambda phage by the procedure of Hohn (10). *E. coli* HB101 was infected with packaged DNA, and cells were plated on L agar containing kanamycin and tetracycline to select for recombinant plasmids containing Ω3080 and flanking sequences. Plasmids were purified from Tet^r Kan^r strains by the method of Birnboim and Doly (3) and analyzed by standard restriction endonuclease digestion and agarose gel electrophoresis procedures (6). A clone which contained Ω3080 flanked by *M. xanthus* sequences inserted at the *EcoRI* site of pHC79 (pKO2; see Fig. 1) was obtained.

pKO2 was subcloned into three smaller plasmids (see Fig. 1). pKO22 was constructed by digestion of pKO2 with *HindIII*, religation, and selection for Amp^r Kan^r transformants. pKO27 was obtained by digestion of pKO2 with *PstI*, religation, selection for Kan^r Tet^r transformants, and screening of these transformants for Amp^s Kan^r Tet^r colonies.

pKO23 was obtained by digestion of pKO2 with *Pst*I, religation, selection for Tet^r transformants, and screening of these transformants for Amp^r Kan^r Tet^r colonies. The structures of all plasmids were confirmed by standard restriction endonuclease digestion and agarose gel electrophoresis techniques (6).

After this study was begun, a plasmid containing the *attP* site of the myxophage Mx8 was constructed for use in genetic analyses of *M. xanthus*. This vector allows the plasmid to integrate at the *attB* site on the *M. xanthus* chromosome (26). Although this vector has many applications, it would not have been appropriate for this study, since we wanted to construct merodiploids in which the 5' and 3' sequences were reconstructed with the tandem duplication.

Tn5 mutagenesis of pKO23. *E. coli* HB101 containing pKO23 was infected with λ ::Tn5. λ ::Tn5 has suppressible mutations in genes required for replication and lytic infection. These mutations are not suppressed in HB101; therefore, λ ::Tn5 does not lyse infected cells, and the DNA is diluted out upon cell division. Infected cells were plated on L agar containing kanamycin. Kan^r colonies were pooled, and plasmids were extracted (3) and transformed into HB101, with selection for Kan^r Tet^r transformants. Plasmids were purified from these transformants and analyzed by standard restriction endonuclease digestion and agarose gel electrophoresis techniques (6) to map the position and orientation of Tn5 transpositions. The distances between insertions and map locations were accurate to within approximately 100 base pairs (bp). Twenty-eight independent transpositions in *M. xanthus* sequences were identified (see Tables 2 and 3).

Bacteriophage P1 lysates and transductions into *M. xanthus*. Plasmids were transformed into *E. coli* C600, and P1 lysates were made at 40 to 42°C as described by O'Connor and Zusman (20). Lysates typically had a titer of 10⁵ plasmid-transducing particles per ml of lysate.

M. xanthus was grown to a concentration of 2 × 10⁸ to 6 × 10⁸ cells per ml in CYE. Cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4°C. Cells were suspended at a concentration of 4 × 10⁹ cells per ml in 10 mM Tris (pH 7.6)–8 mM MgSO₄ (TM buffer). Cells (40 μl) were incubated with 1 to 500 μl of P1 lysate (to give 100 to 500 transductants per plate) for 15 min at room temperature. Infected cells in CF top agar (0.7% agar) were inoculated onto CF agar containing antibiotics. Transductants were incubated at 34°C for 10 to 12 days before fruiting phenotypes were scored under a dissecting microscope at a magnification of ×6.3 to ×16.

Mx4 lysates and transductions. Mx4 plate lysates were prepared as described by Torti and Zusman (28). Transductions were performed as described above for P1 lysates.

Isolation of new Tn5 mutations in *tag* in *M. xanthus*. *M. xanthus* P1 transductants which were Kan^r Tet^r were examined by Southern blot analysis with nick-translated, ³²P-labeled pKO23 as a probe (6). Those strains which were shown to have a single copy of Tn5 in a *tag* gene, no vector sequences, and no wild-type copy of the same *tag* gene were chosen for further study. Only merodiploids were obtained by P1 transduction of plasmid pKO23-1 (Ω361). We therefore made an Mx4 lysate from a merodiploid and used Mx4 transduction to construct a strain of *M. xanthus* containing Tn5 insertion Ω361 (21).

Testing the fruiting phenotypes of the strains. Cells were removed from CYE growth medium by centrifugation at 12,000 × *g* for 10 min at 4°C. The cell pellet was suspended

in TM buffer at 4 × 10⁹ to 8 × 10⁹ cells per ml. Aliquots (5 μl) of cells were dispensed onto CF agar and incubated at 28 or 34°C for 5 days before fruiting morphology was scored. Development at 28°C was compared with development at 34°C. DZF1 was used as the wild-type control in all experiments.

RESULTS AND DISCUSSION

The genetic analyses reported below relied upon the use of P1 to transfer plasmids to *M. xanthus*. Since vector pHC79 does not replicate in *M. xanthus* (unpublished data), vector DNA must become integrated into the *M. xanthus* chromosome for the organism to acquire Tet^r from pHC79. The simplest mechanism by which this occurs is through a single crossover between the *M. xanthus* chromosome and a chimeric plasmid (e.g., pKO2) at a site of homology between the chromosome and the *M. xanthus* sequences of the plasmid. Thus, by selecting for Tet^r Kan^r transductants from P1 lysates carrying any one of the plasmids pKO2, pKO22, pKO27, or pKO23-1 to pKO23-28 (Fig. 1 and 2 and Tables 2 and 3), we obtained merodiploids. These strains contained an entire copy of the transduced plasmid integrated in tandem with the chromosomal gene.

Verification of the cloning of the wild-type alleles of *aggR* mutations. Morrison and Zusman (19) had described a number of apparently temperature-sensitive *M. xanthus* mutants which were unable to complete normal development. Among these mutants were a group which appeared to be unable to aggregate but which were able to sporulate with wild-type kinetics. Torti and Zusman (28) used the myxophage Mx4 (5) to map eight of the mutations to four loci (*aggR1*, *aggR2*, *aggR3*, and *aggR4*) closely linked to the Tn5 insertion Ω3080. It should be noted that DZF3080, containing Ω3080, has a wild-type phenotype for development.

pKO2 was obtained as described in Materials and Methods by the ligation of *Eco*RI-digested chromosomal DNA of DZF3080 into the *Eco*RI site of vector pHC79 (11). To verify that wild-type alleles of the *aggR* loci were present on pKO2, we transduced pKO2 to *M. xanthus* AggR strains by P1 transduction (20) (Table 4). The high rate of cotransduction of pKO2 and wild-type fruiting (Fru⁺) indicated that the wild-type alleles of *aggR1* and *aggR2* were present on pKO2. However, an *aggR3* mutant could not be transduced to Fru⁺ by pKO2; thus, *aggR3* was not obtained in our cloning procedure. pKO2 was subcloned with restriction endonuclease sites in Tn5 to create two plasmids: pKO22 had a deletion of the *M. xanthus* DNA flanking IS50 right of Tn5; pKO27 had a deletion of the *M. xanthus* DNA flanking IS50 left of Tn5 (Fig. 1). The transduction of pKO22 and pKO27 into AggR strains demonstrated that the *aggR* alleles were located in sequences flanking IS50 right of Tn5 (pKO27).

Generating new mutations. pKO23 is a subclone of pKO2 in which the DNA between the *Pst*I site of the vector and the *Pst*I site of IS50 right of Tn5 has been deleted. This clone retains the capacity to render strains Tet^r but no longer renders strains Amp^r or Kan^r because of the partial loss of *bla* and the complete loss of *neo* in the deletion. Since pKO23 was no longer Kan^r, we were able to screen for Tn5 transposition into pKO23 as described in Materials and Methods. The residual DNA from IS50 right of Tn5 adjacent to vector sequences in pKO23 did not appear to interfere with our ability to obtain and identify new transpositions into the plasmid. The use of Tn5 to generate new mutations had three advantages. (i) The mutations were selectable in both

FIG. 1. Chimeric plasmids utilized in the analysis of the *tag* loci of *M. xanthus*. Plasmids were constructed as described in Materials and Methods. pK02 contains an *Eco*RI fragment of DZF3080 cloned into the *Eco*RI site of pHC79. pK022 was obtained by digestion of pK02 with *Hind*III, followed by religation and selection for kanamycin-resistant transformants. pK023 and pK027 were obtained by digestion of pK02 with *Pst*I, followed by religation. pK027 confers resistance to both tetracyclines and kanamycin; pK023 confers resistance only to tetracyclines. pK023-17 is an example of a series of plasmids containing Tn5 insertions in pK023. Insertions were obtained as described in Materials and Methods, and their relative positions were determined by restriction endonuclease analysis. *bla*, β -Lactamase; *neo*, neomycin phosphotransferase; *tet*, tetracycline resistance; H, *Hind*III restriction site; P, *Pst*I restriction site; R, *Eco*RI restriction site.

E. coli and *M. xanthus* through the Kan^r phenotype conferred by Tn5. (ii) The sites of insertion could be physically mapped to the plasmid by standard techniques. (iii) Most, if not all, of the mutations would prohibit the production of a functional gene product from the mutated gene.

One hundred independent transpositions of Tn5 into pK023 were obtained from two trials of the mutagenesis procedure described in Materials and Methods. Of these, 28 were chosen for further study because they represented all regions of the DNA in which transpositions had been identified and because they were all easily distinguishable by standard techniques (6). An example of a Tn5 insertion in pK023 is shown in Fig. 1. The plasmids obtained from this mutagenesis were designated pK023-1 to pK023-28. The

Tn5 insertions were designated Ω 361 to Ω 388, with Ω 361 in pK023-1 and Ω 388 in pK023-28. Ω 361 is the Tn5 insertion closest to the site of the Ω 3080 insertion. The positions of the Tn5 insertions are given in Table 3 and shown in Fig. 3.

Phenotypes of the new mutants. The new Tn5 insertions were transduced into wild-type *M. xanthus* DZF1, which was also the parent strain of the AggR mutants, as described in Materials and Methods. Strains which had acquired Kan^r but which were Tet^s (i.e., had acquired no vector sequences) were analyzed by hybridization to Southern blots (6). Those strains which could be shown to have acquired the mutated form of the gene containing Tn5 with no other detectable changes in the DNA were further analyzed for their ability to develop as described in Materials and Methods. All Tn5

Possible sites of homologous recombination:

Result of homologous recombination at site A:

Result of homologous recombination at site B:

Result of homologous recombination at site C:

FIG. 2. Fate of P1-transduced plasmids in *M. xanthus*. Plasmid-borne genes were stably acquired only through homologous recombination between the recipient chromosome and *M. xanthus* sequences on the plasmid. The presence of both oxytetracycline and kanamycin in the selection medium required that recombination between the plasmid and the chromosome would result in the incorporation of vector sequences in the *M. xanthus* chromosome. This diagram depicts the consequences of a single recombination event between the plasmid and the chromosome with the donor **Tn5** insertion to the right of the **Tn5** insertion in the recipient. A similar diagram can be derived for crosses in which the donor **Tn5** insertion is to the left of the **Tn5** insertion in the recipient.

TABLE 2. Complementation analysis of *tag* by P1 transduction of plasmids from *E. coli* to *M. xanthus*^a

Donor plasmid	Donor Tn5 insert	% of the following total <i>M. xanthus</i> recipients having a Fru ⁺ phenotype:																<i>tag</i>
		DZF1 (wild type)	DZF345 (HA)	DZF3429 (Δ367)	DZF3431 (Δ368)	DZF3439 (Δ369)	DZF3414 (Δ370)	DZF3417 (Δ374)	DZF3418 (Δ376)	DZF3430 (Δ378)	DZF3419 (Δ379)	DZF3420 (Δ381)	DZF3421 (Δ382)	DZF3422 (Δ383)	DZF3424 (Δ385)	DZF3425 (Δ386)	DZF3426 (Δ387)	
pKO23	Δ3080	>97	96	94	31	99	98	46	98	99	98	99	88	62	>99	98	>98	Not <i>tag</i>
pKO23-1	Δ361	ND	<1	91	44	88	96	45	95	>82	90	86	85	29 ^b	98	95	97	<i>tagA</i>
pKO23-2	Δ362	>98	9	<8	36	>83 ^b	95	73	95	97	96	97	83	56 ^b	97	>96	95	<i>tagA</i>
pKO23-3	Δ363	>96	17	54	59	97	98	72	>94	93	95	94	72	45 ^b	99	>90	97	<i>tagA</i>
pKO23-4	Δ364	>95	6	99	58	96	95	67	>94	97	95	97	73	46 ^b	98	>90	>95	<i>tagA</i>
pKO23-5	Δ365	>94	8	<2	42	>92	92	67	88	98	95	95	86	74 ^b	96	>90	94	<i>tagA</i>
pKO23-6	Δ366	94	55	47	41	ND	89	69	97	62	87	94	51 ^b	ND	95	91	95	<i>tagA</i>
pKO23-7	Δ367	92	48	<8	49	90	85	57	93	96	70	85	54	46 ^b	93	93	95	<i>tagB</i>
pKO23-8	Δ368	ND	65	73	<1	72	73	<4	<1	63 ^b	76	58	46	<3	84	81	84	<i>tag?</i>
pKO23-9	Δ369	ND	87	71	47	<0.2	82	<7	<2	73 ^b	81	77	60	13 ^b	89	92	87	<i>tag?</i>
pKO23-10	Δ370	22	45	46	3	39	<1	<14	<20	82	14 ^b	27 ^b	20 ^b	4 ^b	84	71 ^b	86	<i>tagC</i>
pKO23-11	Δ371	94	65	32	33	0.4	1	<4	<4	79	60 ^b	80	17 ^b	11 ^b	77	64 ^b	77	<i>tagC</i>
pKO23-12	Δ372	92	81	<3	3	<3	8	<4	<3	82	79	59 ^b	<1 ^b	15 ^b	83	65	88	<i>tagC</i>
pKO23-13	Δ373	37	42 ^b	16	<2	26	5	<5	<3	53	61 ^b	63 ^b	13 ^b	3 ^b	69	77	76	<i>tagC</i>
pKO23-14	Δ374	35	21 ^b	38	<2	5	22 ^c	<1	<2	64	81	60 ^b	21 ^b	4 ^b	79	71	74	<i>tagC</i>
pKO23-15	Δ375	93	29 ^b	48	<20	<20	<2	<3	<6	20	25 ^b	60 ^b	20	<20	90	15	94	<i>tagC</i>
pKO23-16	Δ376	90	65	53	28	<4	1	<1	<3	6	79 ^b	93 ^b	11	17 ^b	93	92	75	<i>tagC</i>
pKO23-17	Δ377	95	79	59	<5	<2	2	<4	<3	84	78 ^b	76 ^b	3	5 ^b	84	41	88	<i>tagC</i>
pKO23-18	Δ378	97	43	71	50	>86	79	66	98	<5	88	95	95	36	96	99	99	<i>tagD</i>
pKO23-19	Δ379	85	69 ^b	53	14	86 ^b	87 ^b	64	62 ^b	86	<3	<8	<1	<13	77 ^b	77 ^b	92	<i>tagE</i>
pKO23-20	Δ380	80	52 ^b	63	13	56 ^b	76 ^b	27 ^b	90	54	<1	10	<1	1 ^b	72	68 ^b	67	<i>tagE</i>
pKO23-21	Δ381	>98	ND	73	75	82	86	95	94	98	ND	ND	ND	<6	99	95	94	<i>tagE</i>
pKO23-22	Δ382	>99	81 ^b	46	42	67	83 ^b	40 ^b	84	88	<4	<1	<1	<100	88	77	75 ^b	<i>tagE</i>
pKO23-23	Δ383	94	85 ^b	80	33	85	77 ^b	30 ^b	76	90	88 ^c	85 ^c	<2	1 ^b	80	76 ^b	93	<i>tagE</i>
pKO23-24	Δ384	ND	62	67	56	94	88	60 ^b	>94	86 ^b	90	86	47	22 ^b	55	62	62	<i>tagF</i>
pKO23-25	Δ385	>95	74	95	50	98	92	60	97	78	83	97	82	7 ^b	<7	<3	78	<i>tagG</i>
pKO23-26	Δ386	>89	48	87	51	87	87	67	>92	84	60 ^b	44 ^b	67 ^b	<100	<20	<6	64	<i>tagG</i>
pKO23-28	Δ388	ND	ND	ND	>50	ND	33 ^b	<33	>50 ^b	ND	40 ^b	<50	ND	ND	<100	<100	>50	<i>tagI</i>

^a P1 lysates were made on *E. coli* strains carrying each of the plasmids listed. *M. xanthus* was transduced with these lysates and plated on CF agar containing kanamycin and oxytetracycline at 34°C as described in Materials and Methods. Strains and genetic loci are described in Table 1 and in the text. Most transductions were repeated at least three times, and the data were averaged. The positions of the Tn5 insertions are given in Table 3 and shown diagrammatically in Fig. 3. HA, Hydroxylamine mutant; ND, not determined.

^b Fru⁺ colonies had an intermediate fruiting phenotype. Although the cells aggregated and formed fruiting bodies, these fruiting bodies were more diffuse than the wild-type ones and a higher proportion of the spores were found outside the fruiting bodies.

^c Discussed in the text.

TABLE 3. Summary of complementation analyses of *tag* and physical mapping of Tn5 insertion mutations^a

Tn5 insertion	Distance of Tn5 from the end of Ω 3080 (kbp)	<i>tag</i> complementation group
Ω 3080	0	Not in <i>tag</i>
Ω 361	0.172	<i>tagA</i>
Ω 362	1.96	<i>tagA</i>
Ω 363	2.24	<i>tagA</i>
Ω 364	2.58	<i>tagA</i>
Ω 365	3.44	<i>tagA</i>
Ω 366	3.85	<i>tagB</i>
Ω 367	4.23	<i>tag?</i>
Ω 368	4.37	<i>tag?</i>
Ω 369	4.38	<i>tag?</i>
Ω 370	4.39	<i>tagC</i>
Ω 371	4.41	<i>tagC</i>
Ω 372	4.43	<i>tagC</i>
Ω 373	4.80	<i>tagC</i>
Ω 374	5.08	<i>tagC</i>
Ω 375	5.15	<i>tagC</i>
Ω 376	5.20	<i>tagC</i>
Ω 377	5.59	<i>tagC</i>
Ω 378	6.30	<i>tagD</i>
Ω 379	7.00	<i>tagE</i>
Ω 380	7.08	<i>tagE</i>
Ω 381	7.20	<i>tagE</i>
Ω 382	7.33	<i>tagE</i>
Ω 383	7.58	<i>tagE</i>
Ω 384	8.03	<i>tagF</i>
Ω 385	8.12	<i>tagG</i>
Ω 386	8.22	<i>tagG</i>
Ω 387	8.30	<i>tagH</i>
Ω 388	8.50	<i>tagI</i>

^a Tn5 insertions were obtained in pKO23 in *E. coli* HB101 as described in Materials and Methods. Tn5 Ω numbers reflect the stock number of the *E. coli* strain containing the plasmid with the given Tn5 insertion. The positions of the insertions were determined by restriction endonuclease digestion and agarose gel electrophoresis (6). Ω 3080 is the only known linked Tn5 insertion which does not cause the Tag phenotype in *M. xanthus*. The assignment of complementation groups was based upon the data in Tables 2 and 5.

insertions in pKO23 were found to create a temperature-sensitive aggregation phenotype in mutants when the mutations were transduced into DZF1, DZ2, and DK1622 (data not shown).

Complementation analysis of *tag* loci. P1 transductions of Tn5-mutagenized plasmids from *E. coli* to *M. xanthus* were used to construct a map of the complementation groups of *tag*. Transductants were plated on CF agar containing both oxytetracycline and kanamycin to select for *tet* in the vector

TABLE 4. P1 transduction of wild-type cloned *tag* DNA into AggR mutants of *M. xanthus*^a

Recipient strain	Genotype	% of total P1 transductants having a wild-type phenotype (Fru ⁺) with the following donor plasmid:		
		pKO2	pKO22	pKO27
DZF1	Wild type	99	99	99
DZF1516	<i>aggR1</i>	99	2	98
DZF1552	<i>aggR2</i>	95	<0.6	94
DZF1589	<i>aggR3</i>	<1	<2.6	<7

^a P1 lysates were made on *E. coli* strains carrying each of the plasmids (pKO2, pKO22, and pKO27). *M. xanthus* DZF1 was transduced with these lysates and plated on CF agar containing kanamycin and oxytetracycline at 34°C as described in Materials and Methods. Strains and genetic loci are described in Table 1 and in the text.

and *neo* in Tn5, respectively. The CF agar plates allowed us to determine the developmental phenotypes of the transductants, since colonies grew on this medium and, when nutrients were exhausted, entered development in situ (8). Since pHC79 cannot replicate independently in *M. xanthus*, selection for resistance to oxytetracycline recovered transductants in which the plasmid had become integrated in the *M. xanthus* chromosome, forming merodiploids.

Figure 2 is a diagrammatic representation of the results of homologous recombination between the plasmid transduced from *E. coli* and the *M. xanthus* chromosome. It is drawn such that the start of *tag* sequences is located near the *Pst*I site of the vector. Among a number of possible homologous recombination events between the plasmid transduced from *E. coli* and the *M. xanthus* chromosome (20), there are three major merodiploid constructs which can arise. (i) The site of recombination could lie between the vector sequences and sequence 1 (Fig. 2, site A, type I merodiploid). Insertion mutations would be located on both sides of the incorporated vector. Such a strain would have a wild-type phenotype if *M. xanthus* sequences 1 and 2 were separate genes not in the same operon. However, if sequences 1 and 2 were in the same gene or were different genes in the same operon, the phenotype of the strain would be Tag. This prediction is based upon the assumption that Tn5 insertions in *tag* would exert polar effects on downstream genes in the same operon. Although this assumption has not been tested in *tag*, it has been shown to be true for the *frz* operon of *M. xanthus* (30). (ii) If recombination between the plasmid and the chromosome occurred at site B (Fig. 2) lying between sequences 1 and 2, the phenotype of the strain would be wild type whether sequences 1 and 2 were independent genes, two genes in the same operon, or even in the same gene. We know this because the vector is located at the same position as Ω 3080 relative to *tag* and because DZF3080 is wild type for fruiting. Thus, recombination at site B would be sufficient to allow a wild-type phenotype. It should be noted that the frequency of recombination at site B would be very low if sequences 1 and 2 were closely spaced. (iii) If recombination between the plasmid and the chromosome occurred at site C (Fig. 2, type II merodiploid), the strain would have a wild-type phenotype only if sequences 1 and 2 were independent genes not in the same operon.

The results of the complementation analyses are presented in Table 2. There are three salient points to be considered in the interpretation of these data. (i) The proportion of Fru⁺ transductants in the population is affected by the relative frequencies of recombination at sites A, B, and C (Fig. 2). (ii) The relative frequencies of recombination at sites A, B, and C are affected by the positions of the two Tn5 insertions relative to vector sequences and to each other. For example, crossovers at sites A and B should be more common than crossovers at site C when Ω 367 is crossed with Ω 388. Conversely, crossovers at site C might be more frequent than crossovers at site A when Ω 367 is crossed with Ω 368; crossovers at site B should be very rare. (iii) Two crossovers between the chromosome and a multimeric plasmid can construct a merodiploid in which the chromosomal copy of a gene is replaced by the insertion of two copies of the plasmid-borne gene flanking the vector. These transductants will be Fru⁺ and will occur in all crosses. Thus, the proportion of Fru⁺ transductants will not be 100% even when the two mutations in the cross do complement. Nevertheless, examination of a number of transductants from each cross allowed us to designate complementation groups for the *tag* region.

FIG. 3. Positions of Tn5 insertions and tag complementation groups of *M. xanthus*. Tn5 insertions and corresponding *M. xanthus* strains were obtained as described in Materials and Methods. The positions of the Tn5 insertions relative to $\Omega 3080$ were determined by exhaustive restriction endonuclease analysis; the distances between insertions and map locations are accurate to within approximately 100 bp. Complementation data are presented in Tables 2 and 5; the analysis of the data is presented in the text. A to I, tag complementation groups; P, *Pst*I restriction site; R, *Eco*RI restriction site. $\blacksquare$, Position of $\Omega 3080$ (used as a reference point, it is not present in the wild-type chromosome or in Tag strains). Stem and loop structures are numbered 1 to 28. The positions of the Tn5 insertions were determined by restriction endonuclease analysis. kb, Kilobase pairs.

An examination of the data in Table 2 will show that certain reciprocal crosses generated quite different frequencies of Fru^+ transductants. For example, when $\Omega 367$ was crossed with $\Omega 374$, the percentage of transductants which were Fru^+ was dependent upon whether $\Omega 374$ was in the donor plasmid pKO23-14 (38%) or in the recipient chromosome of DZF3417 (57%). Since the position of $\Omega 374$ is near the middle of the cloned tag region and $\Omega 367$ is nearer the *Pst*I site, one might expect there to be slightly more crossovers at site C than at site A. When DZF3417 was the recipient and pKO23-7 was the donor, crossovers at site C constructed type II merodiploids (Fig. 2). However, when DZF3429 was the recipient and pKO23-14 was the donor, crossovers at site C produced type I merodiploids (Fig. 2). Although both type I and type II merodiploids arose from both crosses, there was a bias toward type II merodiploids when $\Omega 374$ was in the recipient chromosome. The data show that there were proportionately more Fru^+ transductants when $\Omega 374$ was the recipient. Significantly, the proportion of Fru^+ transductants did not approximate 100% in either cross. This led us to speculate that type I and type II merodiploids were not functionally equivalent. We tested this hypothesis by constructing a number of deletion plasmids. The only *Pst*I sites in the pKO23::Tn5 plasmids were in the vector and Tn5. Therefore, digestion of the plasmids with *Pst*I and religation of the DNA allowed us to construct plasmids in which all *M. xanthus* DNA between the *Pst*I sites of the vector and Tn5 had been deleted. Consequently, no recombination could occur at site A, effectively eliminating the formation of type I merodiploids but allowing the formation of type II merodiploids. Such plasmids are indicated by the suffix P in Table 5. When $\Omega 374$ was in the recipient chromosome in crosses with deletion plasmids, the percentage of transductants with a Fru^+ phenotype approached 100. This result indicates that type II merodiploids were Fru^+ but that type I merodiploids were not.

Deletion plasmids of the type described above were constructed from pKO23-1, -2, -6 to -12, -14, -18, -19, -23, -24, and -26 to -28. These plasmids were used to clarify the complementation analyses of those reciprocal crosses which had widely divergent frequencies of complementation between the two alleles being studied. In a manner analogous to that described above, we were able to determine which type of merodiploid gave rise to Fru^+ and Fru^- transductants. For example, the data in Table 2 indicated that $\Omega 368$ was in the same complementation group as $\Omega 374$; however, $\Omega 369$ might not be in the same group. Complementation

analysis with the deletion plasmids demonstrated that, in fact, neither $\Omega 368$ nor $\Omega 369$ was in the same complementation group as $\Omega 374$. However, $\Omega 370$ was shown to be in the same complementation group as $\Omega 374$ (Table 5). Similar analyses were done to demonstrate that $\Omega 362$ and $\Omega 367$ were not in a single complementation group (data not shown).

When DZF3414 ($\Omega 370$ on the chromosome) was transduced with pKO23-13 ($\Omega 373$ on the plasmid), 22% of the transductants were Fru^+ . This value is higher than that which would have been expected in comparison with the frequency of Fru^+ transductants for neighboring Tn5 insertions. However, we believe that $\Omega 370$ and $\Omega 373$ are in the same complementation group for the following reasons. (i) The Tn5 insertions flanking $\Omega 373$, $\Omega 372$, and $\Omega 374$ both failed to complement $\Omega 370$. (ii) After numerous efforts, we were unable to construct a strain with $\Omega 373$ on the chromosome. This result indicates that the mutation may have been lethal. In addition, the number of transductants obtained in the cross with pKO23-13 was approximately one-fifth the

TABLE 5. Complementation analysis of $\Omega 374$ with deletion plasmids^a

Donor plasmid	Tn5	% of total transductants which were Fru^+
pKO23-1	$\Omega 361$	45
pKO23-2	$\Omega 362$	73
pKO23-6	$\Omega 366$	69
pKO23-7	$\Omega 367$	57
pKO23-8	$\Omega 368$	<4
pKO23-9	$\Omega 369$	<7
pKO23-10	$\Omega 370$	<4
pKO23-1P	$\Omega 361$	85
pKO23-2P	$\Omega 362$	90
pKO23-6P	$\Omega 366$	>95
pKO23-7P	$\Omega 367$	>95
pKO23-8P	$\Omega 368$	96
pKO23-9P	$\Omega 369$	96
pKO23-10P	$\Omega 370$	<8

^a P1 lysates were made on *E. coli* strains carrying each of the plasmids listed. *M. xanthus* DZF3417 was transduced with these lysates and plated on CF agar containing kanamycin and oxytetracycline at 34°C as described in Materials and Methods. Strains and genetic loci are described in Table 1 and in the text. The data reported for plasmids pKO23-1, pKO23-2, pKO23-6, pKO23-7, pKO23-8, pKO23-9, and pKO23-10 are the same as the data in Table 2; they are repeated here for ease of comparison to the data for the deletion plasmids. The positions of the Tn5 insertions are given in Table 3 and shown diagrammatically in Fig. 3. The construction of the deletion plasmids is described in the text.

number of transductants obtained in other crosses with DZF3414 as the recipient. We have already noted that type I merodiploids were not Fru⁺ in this complementation group. Perhaps in this particular cross, type I merodiploids were lethal rather than merely Fru⁻. In that case, Fru⁺ transductants arising from recombination at site B (Fig. 2) would constitute a proportionately larger share of the viable transductants.

There were also a very large number of Fru⁺ transductants in the crosses of pKO23-23 (Ω383) with DZF3419 (Ω379) and with DZF3420 (Ω381). The reciprocal crosses gave no Fru⁺ transductants. Crosses between either DZF3419 or DZF3420 and the Ω379 deletion plasmid (pKO23-13P) also gave only Fru⁻ transductants. In addition, we also constructed a deletion plasmid from pKO23 in which all *M. xanthus* DNA between a *Xho*I site in the vector and a *Sal*I site 100 bp to the left of the wild-type site of Ω379 had been deleted. pKO28 carried the corresponding wild-type sequences for Ω379 to Ω388 but did not carry the wild-type sequences for Ω361 to Ω378 (*tagA* to *tagD*). When pKO28 was crossed with DZF3422 (Ω383 on the chromosome), only Fru⁻ transductants were obtained. These transductants were wild type for *tagA* to *tagD*, wild type at the site of the Ω379 insertion, carried the Ω383 insertion, and carried the corresponding wild-type sequence at Ω383 (on integrated pKO28). The Fru⁻ phenotype of these strains indicates that sequences to the left of Ω379 (Fig. 3) are required to complement Ω383. pKO28 also failed to complement Ω379 to Ω382 (strains DZF3419, DZF3420, and DZF3422) but did complement Ω386 (strain DZF3424) with a frequency of 100%. These data led us to place Ω383 in the same complementation group with Ω379 to Ω382. The high incidence of Fru⁺ merodiploids in crosses between DZF3419 and DZF3420 with pKO23-23 may reflect a strong selection for transductants with crosses at site B (Fig. 2).

After dealing with complications surrounding the insertion mutations Ω370 to Ω377 and Ω379 to Ω383 as described above, we assigned the mutations created by Tn5 insertions Ω361 to Ω388 to nine complementation groups (Table 3). The number of new mutations generated and the number of complementation groups identified for these mutations made the previous *aggR* notation adopted by Torti and Zusman (28) unworkable. *aggR1* was quickly recognized to be representative of the third complementation group distal to Ω3080; *aggR2* was found to be representative of the fifth complementation group of a total of nine complementation groups distal to Ω3080. We therefore found it necessary to use a different notation to identify the mutations presented in this paper. *tag* was chosen because mutants were found to be temperature dependent for aggregation. Several mutations remain unassigned: Ω367, Ω368, and Ω369 do not map into a complementation group but are physically separated by less than 200 bp. They are not in the same complementation group as Ω374 (Table 5), but complementation was nevertheless very low in crosses with members of *tagC*. In addition, five complementation groups were defined by single insertion mutations: *tagB*, *tagD*, *tagF*, *tagH*, and *tagI*.

It should be noted that, although the data do define complementation groups, they do not define individual genes. For example, *tagA* is quite large (possibly as large as 3.8 kbp) and may contain several genes under the control of a single promoter. Other complementation groups are relatively small (the maximum possible sizes are 0.8 kbp for *tagB*, 0.6 kbp for *tagF*, 0.3 kbp for *tagG*, and 0.3 kbp for *tagH*). These small complementation groups may reflect

individual functional domains of larger, multifunctional proteins.

Partial complementation of the Tag phenotype. We now direct our attention to another phenomenon which occurred in these analyses: partial complementation. In a number of crosses, especially those between complementation groups *tagC* and *tagE*, transductants were not completely rescued to wild-type fruiting. Fru[±] (partially complemented) colonies made fruiting bodies which were flatter than wild-type ones, and more spores were found among the peripheral rods. These strains retained their phenotype when retested on CF agar—i.e., Fru[±] was a stable phenotype (data not shown). Partial complementation was considered to be equivalent to full complementation for the purposes of defining complementation groups. However, partial complementation indicated that the mutated *tagC* and *tagE* genes interfered with the action of the wild-type *tagC* and *tagE* genes. Such an interaction could be at the level of gene regulation or at the level of the interaction of two polypeptides in their function(s) in the cell.

The question arises as to whether the phenomenon of partial complementation was due to the polar nature of Tn5 insertions. We addressed this question by performing complementation analyses of point mutations in the *tag* region. The mutants were isolated by Morrison and Zusman (19) and by Torti and Zusman (28). We found that DZF1516 is in *tagC* and that DZF1552 is in *tagE* (Table 6). In addition, all four *tagC* point mutations were only partially complemented by *tagE* mutations. All three *tagE* mutations were only partially complemented by *tagC* mutations. These results with point mutations and the fact that Ω378 fully complemented both *tagC* and *tagE* indicated that the polar nature of the Tn5 insertion mutations was not responsible for the phenomenon of partial complementation.

We note that, like full complementation, partial complementation also varied between reciprocal crosses. For example, with Ω374 in the recipient chromosome, 95% of the transductants were Fru⁺, with few Fru[±] (partially complemented) transductants. Most merodiploids would be of type II or would have recombined at site B (Fig. 2). With Ω374 on the donor plasmid, more merodiploids would be of type I than of type II, with the frequency of recombination at site B remaining the same as with Ω374 in the recipient chromosome. However, many of the 63% complemented transductants were only partially complemented. This result suggests that type I merodiploids were partially functional but that type II merodiploids were fully functional when there were mutations in *tagC* and *tagE*. This suggestion is consistent with the results presented in Table 5, which indicated that type II merodiploids were fully functional while type I merodiploids were not.

The nonequivalence of type I and type II merodiploids suggests that there are sequences distal to either end of the *M. xanthus* DNA cloned in pKO23 which must be contiguous with the cloned sequences to allow wild-type expression of the cloned genes. We note that the vector itself acts as an insertion mutation in the complementation analyses presented in Tables 2, 5, and 6. It interrupts the gene distal to *tagI* and also interrupts the gene prior to *tagA*. We have stated elsewhere in this paper that the vector is inserted upstream of *tagA* at precisely the location of Ω3080. Since DZF3080 makes wild-type fruiting bodies at 34°C, we have assumed that Ω3080 lies outside *tag*. However, Ω361 (*tagA*) lies within 200 bp of Ω3080. It is possible that Ω3080 is close enough to *tag* to have deleterious effects on fruiting if another mutation is also in the cell but that the effects of

TABLE 6. Complementation analyses of AggR mutants^a

Donor plasmid	Donor Tn5 insert	% of the following total <i>M. xanthus</i> recipients having a Fru ⁺ phenotype:						
		DZF1516 (aggR1)	DZF3144 (aggR1)	DZF3159 (HA)	DZF3348 (HA)	DZF3157 (HA)	DZF3163 (HA)	DZF1552 (aggR2)
pKO23	Ω3080	98	99	87	86	98	95	95
pKO23-1	Ω361	89	88	63	76	67	90	>67
pKO23-2	Ω362	90	97	81	84	88	85	85
pKO23-3	Ω363	89	97	76	91	91	81	92
pKO23-4	Ω364	92	95	90	65	83	71	93
pKO23-5	Ω365	86	75	78	71	>75	74	69
pKO23-6	Ω366	89	82	77	90	54 ^b	78 ^b	70
pKO23-7	Ω367	96	81	76	93	90 ^b	91 ^b	83
pKO23-8	Ω368	94	54	20	23	83	81	48
pKO23-9	Ω369	3	1	18	ND	92	91	70
pKO23-10	Ω370	<20	<8	3	8	17 ^b	57 ^b	5 ^b
pKO23-11	Ω371	7	<2	16	<9	25 ^b	34 ^b	44 ^b
pKO23-12	Ω372	7	5	7	6	30 ^b	78 ^b	30 ^b
pKO23-13	Ω373	16	19	2	ND	42 ^b	47 ^b	10 ^b
pKO23-14	Ω374	<2	ND	<3	<4	25 ^b	38 ^b	14 ^b
pKO23-15	Ω375	9	3	<6	9	25 ^b	83 ^b	40 ^b
pKO23-16	Ω376	13	25	<3	13	40 ^b	87 ^b	48 ^b
pKO23-17	Ω377	8	7	<14	<13	59 ^b	63 ^b	21 ^b
pKO23-18	Ω378	98	88	90	78	97	91	99
pKO23-19	Ω379	88 ^b	67	45 ^b	50	<13	<7	11
pKO23-20	Ω380	69 ^b	62 ^b	28 ^b	9 ^b	13	<8	<6
pKO23-21	Ω381	88	86	60	75 ^b	<20	ND	ND
pKO23-22	Ω382	96 ^b	95	35 ^b	61 ^b	4	2	<20
pKO23-23	Ω383	69 ^b	88	51 ^b	76 ^b	70	<3	5
pKO23-24	Ω384	88	88	58	84	87	91	67
pKO23-25	Ω385	93	92	74 ^b	85	89	97	>83
pKO23-26	Ω386	91	86	52 ^b	56 ^b	83 ^b	91 ^b	50
pKO23-28	Ω388	>50	ND	50	ND	ND	>67 ^b	ND

^a P1 lysates were made on *E. coli* strains carrying each of the plasmids listed. *M. xanthus* was transduced with these lysates and plated on CF agar containing kanamycin and oxytetracycline at 34°C as described in Materials and Methods. Strains and genetic loci are described in Table 1 and in the text. Most transductions were repeated at least three times, and the data were averaged. The positions of the Tn5 insertions are given in Table 3 and shown diagrammatically in Fig. 3. HA, Hydroxylamine mutant; ND, not determined.

^b Fru⁺ colonies had an intermediate fruiting phenotype. Although the cells aggregated and formed fruiting bodies, these fruiting bodies were more diffuse than the wild-type ones and a higher proportion of the spores were found outside the fruiting bodies.

Ω3080 in an otherwise wild-type cell are too subtle to have been observed during our experiments. We also have found a Tn5 *lac* insertion (13) in *tagA* which indicates that the direction of transcription is from *tagA* toward *tagB* (unpublished data). Thus, it is conceivable that Ω3080 may lie in a position to affect the transcription of *tagA*.

Regulation of *tag*. The evidence presented in this paper does not address whether *tag* is thermoregulated. However, the aggregation system complementary to *tag* may well be thermoregulated. This complementary system, although sufficient for aggregation at 28°C, is certainly not sufficient for aggregation at 34°C. There are at least three possible patterns for the regulation of the two aggregation systems in *M. xanthus*. (i) Both systems might be functional at 28°C while *tag* is required only at 34°C. (ii) *tag* might be expressed only at 34°C while its complement is expressed only at 28°C. (iii) Both aggregation systems might be expressed at all temperatures, but the ability of the systems to function might be thermoregulated posttranscriptionally. There are examples in the literature for all three regulatory schemes. (i) DegP, a cell envelope protease, is required for growth only at elevated temperatures (42°C) by *E. coli*. However, DegP is synthesized at 37°C as well as 42°C, at least in maxicells (27). (ii) Protein 1, an important factor in the pathogenicity of *Yersinia enterocolitica*, is expressed only at higher temperatures. This thermoregulation remains intact when the gene for protein 1 is present on a plasmid in *E. coli* (16). (iii) The ciliated protozoan *Tetrahymena thermophila* has five immu-

nologically distinct serotypes, three of which are thermoregulated and encoded by separate genes (2). Studies on the regulation of *serH* have demonstrated that the abundance of SerH is regulated posttranscriptionally through the stability of mRNA: SerH mRNA has a half-life of more than 1 h at permissive temperatures, while the half-life of the mRNA is less than 3 min at nonpermissive temperatures (15). Preliminary evidence from the study of a Tn5 *lac* insertion (13) in *tagA* suggests that *tagA* is transcribed at both 28 and 34°C, ruling out a mutually exclusive pattern of regulation for the two aggregation systems in *M. xanthus* (unpublished data).

Summary. The *tag* mutants were first isolated as point mutations which were temperature sensitive for the ability to form fruiting bodies. In this paper we have described the complementation analysis of 28 Tn5 mutations and eight point mutations in *tag*. All but three of these mutations have been assigned to one of nine complementation groups. We began this study as a means of obtaining more selectable mutations in *tag* loci. We anticipated that most of the Tn5 insertions would create mutations which would render cells unable to aggregate at any temperature. We did not expect to pick up any temperature-sensitive mutations. After the first few Tn5 mutations were analyzed, it became obvious that all were temperature dependent and that *tag* was not required for aggregation at 28°C. In the course of constructing the Tn5-mutagenized strains of *M. xanthus*, we found strains with major deletions, duplications, inversions, or other rearrangements of the *tag* region. We also transduced the

tag mutations into two other wild-type strains, DZ2 and DK1622. All mutants were temperature dependent for aggregation (unpublished data).

Thus, through mutational analysis, *tag* has allowed us to demonstrate that there are at least two aggregation systems in *M. xanthus*: one is required at 34°C (*tag*) and the other is sufficient for aggregation at 28°C. Recent work in our laboratory has shown that the two aggregation systems can also be observed by their ability to function during the reinitiation of development following the plating of harvested peripheral rods (a newly described developmental cell type [23]) on fresh developmental medium (unpublished data). The two systems must respond to a similar signal (starvation), but the context of the signal (temperature) determines which system is functional in the cell. Preliminary studies indicate that cell density and nutrition may also influence the determination of which system functions during aggregation. We believe that the two aggregation systems of *M. xanthus* will prove to be a valuable model for the study of gene regulation.

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