Mutations affecting germination in *Myxococcus xanthus*

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*Myxococcus xanthus* mutants defective in myxospore germination have been isolated both by a selective and by a non-selective method after UV or Tn5-lac-induced mutagenesis. The ability of these mutants to germinate in germinant solutions other than those used for their isolation has been tested. Six of seven mutants isolated behaved as germination-defective in all germinants. Germination of the seventh mutant was conditional on the germinant used, being normal in Casamino acids but defective in a Casitone-based medium. Genetic analysis of the four mutant strains carrying Tn5-lac insertions revealed that the transposon had disrupted a different locus in each mutant, so that the four mutants defined four unlinked loci involved in the germination process (gerA, gerB, gerC, gerD). Strain MR307 was studied in more detail. Cloning of the gene affected in this mutant, gerC, and construction of merodiploids revealed that the wild-type allele is dominant over the mutated one. In vitro construction of lacZ fusions allowed study of gerC expression throughout the *M. xanthus* life cycle, revealing that the gene affected by insertion at ΩMR307 is developmentally regulated.

**Introduction**

*Myxococcus xanthus* is a Gram-negative soil bacterium that undergoes a multicellular cycle of development when subjected to starvation on a solid surface. Vegetative swarms migrate towards centres of aggregation, forming raised mounds of cells (fruiting bodies). Within these mounds individual cells differentiate into spherical, environmentally resistant myxospores. When nutrient limitation ends, germination is triggered and myxospores reconvert to vegetative cells (Zusman, 1984; Dworkin & Kaiser, 1985). *M. xanthus* is an excellent bacterial system for the investigation of problems related to the regulation of development (see reviews by Dworkin & Kaiser, 1985; Shimkets, 1987). Recent development of the necessary genetic tools has allowed identification and cloning of loci associated with fruiting and sporulation (Kaiser, 1984).

We have undertaken the genetic analysis of germination in *M. xanthus*. In an accompanying paper (Elías & Murillo, 1991) we report a physiological study on germination of wild-type myxospores carried out to facilitate the search for mutants affected in germination. A collection of germination mutants would make it possible to estimate the number of genes involved as well as whether induction by different germinants proceeds through different germination pathways. Here we describe two methods for isolating germination-defective (Ger-) mutants and report the characterization of several *M. xanthus* mutants. At least four loci affecting myxospore germination have been identified (gerA, gerB, gerC and gerD). We also report the cloning of the gerC locus in a vector that replicates in *Escherichia coli* and the construction of gerC<sup>+</sup>/gerC<sup>−</sup> partial diploids by P1 specialized transduction (Shimkets et al., 1983) of the chimeric plasmid from *E. coli* to *M. xanthus*.

**Methods**

Bacterial strains, plages and plasmids. *M. xanthus* DK1050 (Ruiz-Vázquez & Murillo, 1984) was the parental wild-type germination-competent (Ger<sup>+</sup>) strain. All Ger<sup>−</sup> mutants were obtained by UV or Tn5-lac mutagenesis of this strain. The symbol Ω followed by identification letters and numbers is used to denote a transposon insertion site (see Avery & Kaiser, 1983). In situ replacement of the kanamycin resistance marker (Km<sup>+</sup>) of Tn5-lac insertions by oxytetracycline resistance (Te<sup>+</sup>) was carried out as described by Avery & Kaiser (1983).

Phage Mx4-LA27 was used for generalized transduction between *M. xanthus* strains (Avery & Kaiser, 1983) and coliphage P1 clp-100 cam (Rosner, 1972) was used to transduce cloned DNA from *E. coli* to *M. xanthus* as described by Shimkets et al. (1983).

The cloning vectors pDAH250 and pDASH16 derive from pREG429, a plasmid that carries the Km<sup>+</sup> gene and most of the ISS5L element of Tn5, and a DNA fragment encoding P1-specific incompatibility to efficiently transfer the plasmid by P1 specialized transduction (Gill et al., 1988). Plasmid pDAH156 was used for in vitro construction of lacZ fusions. The three plasmids were constructed and kindly provided by D. A. Hodgson, University of Warwick, UK.

Culturing conditions and preparation of myxospores. *M. xanthus* was grown vegetatively in CTT broth (Hodgkin & Kaiser, 1977). Media derived from CTT by lowering the concentration of Bacto-Casitone are referred to as 1/2 CTT, where 1/2 represents the fraction of Bacto-
Casitone in the medium with respect to that in CTT (10 mg ml⁻¹). Development was induced on CF agar plates (Hagen et al., 1978). Clonal fruiting bodies for germination analysis were obtained by plating single cells on CF agar. Fruiting bodies from which myxospores were harvested were obtained from drops of liquid cultures placed on CF agar. Myxospore suspensions were obtained after sonic disruption of collected fruiting bodies (for details, see Elias & Murillo, 1991).

**Mutagenesis and mutant isolation.** UV mutagenesis was carried out by exposing 3 ml of an exponential culture (at a cell density of 10⁷ cells ml⁻¹) of DK1050 in CTT broth to irradiation for 90 min with a germicidal lamp (General Electric, G15T8), a treatment that yielded around 5% survival. Cells were then pelleted by centrifugation (3000 g, 10 min), suspended in fresh CTT broth and allowed to grow overnight before plating on CF agar. For Tn5-lac mutagenesis, strain DK1050 was infected with P1::Tn5-lac and Km³ transductants were selected as described by Kroos & Kaiser (1984).

Germination-defective mutants were isolated by two methods. The first method allows screening for mutants directly on CF agar. Single cells were plated on CF agar, where they first formed colonies and then fruiting bodies. Plates containing clonal fruiting bodies were incubated at 30 °C for 90 min to kill vegetative cells and then each plate was overlaid with 10 ml 1/3 CTT soft agar. After incubation at 33 °C for 24 h, a clone defective for germination among several wild-type clones was easily recognized as showing much less growth or no growth at all. Putative Ger⁻ clones were toothpoked on to CTT plates and those that grew on this medium were tested again for ability to germinate.

In the second method, myxospores were incubated in a germinant solution (2 g Casamino acids l⁻¹ or 1/30 CTT) for 24 h, which is long enough to ensure germination of wild-type spores, and selection against germinated cells was performed at 50 °C for 90 min. Then germination and growth in CTT was allowed. Conditional Ger⁻ mutants were isolated after two cycles of selection.

**Expression of β-galactosidase.** Rapid determination of β-galactosidase production during growth and development was carried out by examining colony colour on CTT plates containing 40 µg 5-bromo-4-chloro-3-indoly-β-D-galactoside (X-Gal) ml⁻¹ (CFT X-Gal) and on CF plates containing 20 µg X-Gal ml⁻¹ (CF X-Gal).

Disruption of vegetative cells by sonication and measurement of specific β-galactosidase activity were carried out as previously described (Balsalobre et al., 1987). To assay activity from late-developmental or germinant samples, which contain sonication-resistant cell forms, the sonicate treatment was performed in the presence of 0.25 mm glass beads (1 vol. beads per 3 vols cell suspension). The cell debris and glass beads were pelleted before activity of β-galactosidase was determined. Units of β-galactosidase specific activity are nmol o-nitrophenol (ONP) produced min⁻¹ (mg protein)⁻¹.

**Cloning of the gerC locus.** The cloning procedure is illustrated in Fig. 1. Vector pDAH250 was used to construct the 16-kb chimeric plasmid pME100, following the cloning strategy described by Gill et al. (1988) and illustrated in Fig. 1(a). Plasmid pME100 carries a 4.4-kb BamH I fragment adjacent to the right ISS50 element at OMR307. The complete gene was cloned using vector pDAH160, kindly provided by D. A. Hodgson. This plasmid was designed to enable cloning of M. xanthus DNA previously cloned in pDAH250 in reversed orientation with respect to the Km³ gene. As shown in Fig. 1(b), plasmids pDAH160

the Km³ marker. Plasmid pME300 was transduced into DK1050 with selection for Km³ and, after KpnI digestion and transformation of E. coli, the chimeric plasmid pME600 was obtained. This plasmid contained, in addition to the 4.4-kb segment previously cloned in pME100, 6.6 kb of chromosomal DNA (thin line) to the right of OMR307. B, H, K and X are abbreviations for restriction enzymes BamH I, HindIII, KpnI and XhoI, respectively.
plasmid pME100 was treated with XhoI and the four DNA fragments (A, B, C, D) obtained were electrophoresed on low-melting-temperature agarose. The three fragments containing M. xanthus DNA, fragments A (0.93 kb), B (1.43 kb) and C (4.05 kb), were purified from the gel and separately ligated to SauI linearized pDAH156 (Maniatis et al., 1982). The ligations were used to transform E. coli MC1061 to KmR. Stocks of P1 grown on individual KmR transformants carrying the desired chimeric plasmids were used separately to transduce M. xanthus DK1050 with selection for KmR.

**Results and Discussion**

Isolation of Ger- mutants by the in situ assay

An in situ assay has been developed to screen for germination-defective mutants directly on CF plates. Basically, spores developed on CF are induced to germinate by overlaying the plates with 1/3 CTT soft agar. This particular concentration was the lowest concentration of Bacto-Casitone that induced in situ germination of the wild-type and at the same time supported growth of the germinated cells. Following this procedure we isolated six Ger- mutants. Four (MR303- MR306) were obtained after UV irradiation and screening of 4000 spore-containing colonies. The other two mutants, MR301 and MR302, were obtained following Tn5-lac mutagenesis and screening 2000 KmR colonies.

The ability of these mutants to germinate in CTT, 1/4 CTT, 1/10 CTT and 1/30 CTT was quantitatively compared to that of the parental strain DK1050. Germination of strains MR301-MR306 was hardly defective in CTT, but as the concentration of Casitone in the germinant solution was decreased the Ger- phenotype became evident. Germination of strains MR303- MR306 in 1/30 CTT was followed by microscopic observation and by the loss of heat resistance (Fig. 3). The four mutants germinated poorly at this concentration of Casitone. After 14 h incubation in 1/30 CTT (long enough to observe complete germination of wild-type spores), 75–95% of the spore population for the mutant strains was still refractile and round (Fig. 3a). The germination-defective phenotype was also conspicuous when the germination kinetics were estimated as a percentage of heat-sensitive spores (Fig. 3b). As shown for the parental strain (Elias & Murillo, 1991), loss of heat resistance of MR303-MR306 spores occurred much faster than germination assayed by microscopic observation. Strains MR301 and MR302 behaved in a similar way (data not shown).

The six mutants were tested for their ability to germinate in Casamino acids (2 g l⁻¹) and some defined mixtures of amino acids, previously found to permit germination of wild-type myxospores (Elias & Murillo,
All of them showed defective germination behaviour in these solutions.

The specific defect that causes this generalized Ger− phenotype has not yet been characterized. Growth rate and plating efficiency both in CTT and in minimal medium (Bretschler & Kaiser, 1978) for all six mutants was the same as for the wild-type, so a general metabolic defect or an auxotrophy seems unlikely. Under the light microscope, the mutant myxospores appear normal as far as refractivity, shape and size are concerned, and fruiting follows the normal course.

The Km R determinant at Tn5-lac insertion sites ΩMR301 and ΩMR302 provides a useful genetic marker for transductional analysis. The Km R at ΩMR301 and ΩMR302 was separately transduced into the parental strain DK1050. In both cases, 40 transductants were tested by the in situ assay and they all showed the same Ger− phenotype as the corresponding donor strain. This confirms that the germination defect in strains MR301 and MR302 is the result of an insertional mutation. To study linkage between the two insertions, a cross using MR301 as donor and a Tc R replacement of MR302 as recipient was performed. No Km R Tc R transductants were found among 300 Km R colonies tested, thus indicating that the Tn5-lac insertion sites involved map at different positions on the chromosome. The genes affected by the transposon insertion at ΩMR301 and ΩMR302 have been designated gerA and gerB, respectively.

Transposon Tn5-lac, constructed by inserting a promoterless trp− lac fusion fragment near the left end of Tn5, was designed to make transcriptional fusions to exogenous promoters (Kroos & Kaiser, 1984). Expression of the lacZ gene from a Tn5-lac insertion can be readily assayed by measuring β-galactosidase. To study the regulation of the genes gerA and gerB, β-galactosidase activity was determined for strains MR301 and MR302 during vegetative growth, development and germination. Very low specific activity of β-galactosidase was found throughout the life cycle for both strains, as would be expected if Tn5-lac had inserted in the wrong orientation to make a fusion.

Isolation of Ger− mutants by a selective procedure

A selective method for isolating germination mutants was developed based on the possibility of selecting ungerminated spores, which are heat-resistant, from spores that have germinated successfully. Two mutant strains, MR307 and MR308, were independently isolated after insertional mutagenesis with Tn5-lac and two cycles of selection.

Strain MR307 was selected as being incapable of germinating in Casamino acids (2 g l−1) but still responsive to CTT. Germination of MR307 in Casamino acids (2 g l−1) and in CTT was quantified microscopically and by the loss of heat resistance and compared with the germination of the wild-type strain DK1050 (Fig. 4). In
Casamino acids, no myxospore germination was observed up to 24 h. As expected, given the method used for isolation, the loss of heat resistance after such prolonged incubation was hardly appreciable (Fig. 4a). In CTT, spores of MR307 germinated more slowly than the wild-type. This defective response was particularly clear when germination was followed microscopically (Fig. 4b). In addition, MR307 failed to germinate in 1/30 CTT, in a mixture of lysine and histidine (each 1 g l\(^{-1}\)) which induces germination of the wild-type strain (see Elias & Murillo, 1991), and at higher or lower concentrations of Casamino acids than 2 g l\(^{-1}\) (0.5–10 g l\(^{-1}\)).

The other mutant, MR308, was selected to show an altered response for germination in 1/30 CTT but not in CTT. We compared germination of MR308 in each germinant solution with germination of DK1050 (Fig. 5). MR308 was almost as responsive to CTT as the parental strain, when germination was assayed either microscopically or by the loss of heat resistance (Fig. 5a). In 1/30 CTT, spores of strain MR308 germinated considerably more slowly than spores of DK1050. After 24 h incubation, 60% of the mutant spores were still heat-resistant and around 80% had not yet shown any visible morphological changes (Fig. 5a). The germination efficiency of MR308 spores dropped rapidly as the concentration of Casitone was progressively lowered from CTT to 1/30 CTT (not shown). For germination in Casamino acids, however, MR308 showed a behaviour close to the wild-type (not shown). The fact that germination of MR308 is conditional on the germinant used might mean that spores of *M. xanthus* have germination-specific reception systems that can be affected independently, or that different germination pathways exist. Consequently, strain MR307 and the six mutants isolated by the preceding method (MR301–MR306), all defective for germination in CTT and Casamino acids, might be affected in a gene whose function is common to both reception/germination pathways. Alternatively, their mutant phenotype might be indirectly related to germination and be the consequence, for example, of alterations in the spore structure that supports the germination mechanism.

The only alteration so far detected for mutant strains MR307 and MR308 is at the level of germination. Loss of heat resistance is one of the first events during germination (Elias & Murillo, 1991). Spores of MR307 and MR308 remain heat-resistant after prolonged incubation in Casamino acids and 1/30 CTT, respectively. This behaviour, for which they were selected, indicates that both mutants are blocked at an early stage of germination.

Crossovers between the mutants and the parental strain were carried out to confirm that their germination behaviour was due to an insertional mutation. The possibility that these two new mutations mapped within one of the previously identified loci or were linked to each other was also studied. Analysis of the antibiotic resistances of 300 transductants from a cross between MR307 and a Te\(^{r}\) replacement of MR308 revealed that Tn5-lac insertions ΩMR307 and ΩMR308 are located in separate unlinked loci. Neither did we find linkage between these mutations and genes *gerA* and *gerB*. The genes affected by insertion of Tn5-lac at ΩMR307 and ΩMR308 have been called *gerC* and *gerD*, respectively. The germination mutants isolated to date are therefore not clustered and represent at least four different loci.

Expression of β-galactosidase was measured for MR307 and MR308 during vegetative growth, development and germination (Fig. 6). Specific activity of β-galactosidase remained very low for all samples of strain MR307, probably due to the insertion of Tn5-lac in the wrong orientation to allow fusion of lacZ expression to the corresponding promoter. Strain MR308 showed a gradual increase of β-galactosidase activity during vegetative growth. When induced to develop, expression of β-galactosidase in MR308 was still high a few hours after cells were spotted on CF medium, though lower than at t = 0, and continued decreasing throughout development. During germination, β-galactosidase activity remained constant at around 10 units, which is late.
Fig. 6. Expression of β-galactosidase during vegetative growth (a), development (b) and germination (c) of strains MR307 (▲) and MR308 (□).

Similar to the level found at early exponential growth and late development. Thus, insertion of Tn5-lac at ΩM308 is affecting a gene that is expressed throughout the M. xanthus life cycle, showing maximal levels of expression during late exponential growth and early development. These expression profiles would agree with any of the alternative explanations proposed to account for its particular Ger− phenotype.

Cloning the gerC locus

Among the mutants isolated, MR307 showed the strongest Ger+ phenotype. As a step towards the investigation of the function of the affected locus in germination, we cloned the gerC+ allele. Because the mutation in MR307 results from disruption of the gene gerC by insertion of Tn5-lac, a two-step procedure was necessary to clone the wild-type allele. First, the chimeric plasmid pME100 was constructed, according to the cloning strategy described by Gill et al. (1988). This plasmid carried a 4.4-kb BamHI fragment flanking ΩM307, and presumably a truncated gerC allele (Fig. 1a). Cloning of the full-length allele was then attempted following the procedure depicted in Fig. 1(b) (see Methods). Taking advantage of sequence homology provided by pME100, a new chimeric plasmid (pME400) was constructed. This plasmid carried an 11-kb BamHI KpnI fragment spanning 4.4 kb on one side of ΩM307 and 6.6 kb on the other, and was therefore very likely to include the complete gerC locus.

To verify that pME400 carries the gerC+ allele, the plasmid was transduced from E. coli to M. xanthus MR311 selecting for Km8, and rescue of the germination defect was determined. If pME400 carried the complete gerC locus, integration of the plasmid would generate a duplication with a wild-type gerC allele on one copy of the duplication and a mutant allele on the other (see Fig. 7). Germination of 14 Km8 Tc8 transductants in Casamino acids and CTT was tested. Plasmid pME400 restored germination in four such transductants, indicating that pME400 carries the gerC locus and that the gerC+ allele is dominant over the mutant allele. Those transductants expected to have the same gerC+/gerC− duplication that, however, showed a defective phenotype, probably arose by gene conversion resulting in two identical mutant alleles. High gene conversion frequencies in M. xanthus have also been observed by other groups of workers (Shimkets et al., 1983; Stephens & Kaiser, 1987), even for mutations caused by a transposon insertion (M. Fontes, personal communication).

Construction of in vitro lacZ fusions to study expression of the gerC gene

Because insertion of Tn5-lac at ΩM307 seems to have occurred in the wrong orientation to make a fusion, expression of the gerC gene was determined using a
by homologous recombination results in tandem duplication of those sequences common to the insert and the chromosome.

Transductants with plasmids carrying fragment A were not obtained. This may be attributed to the size of *M. xanthus* DNA in this particular insert being too small to allow homologous recombination.

Five Km\(^{R}\) transformants carrying a plasmid with insert B were lysed with coliphage P1 and the chimeric plasmids were independently transduced into DK1050. The incoming plasmid integrates into the chromosome by homologous recombination, duplicating in tandem fragment B. Two alternative constructions are expected depending on the orientation of fragment B with respect to the lacZ gene in the chimeric plasmid (see Fig. 8). Individual Km\(^{R}\) transductants were toothpicked onto nutrient and starvation agar plates containing X-Gal and screened for expression of \(\beta\)-galactosidase. All the transductants obtained in one of the crosses were phenotypically Lac\(^{-}\) as indicated by the absence of a colour reaction on both media. The other four lysates gave rise to transductants that expressed \(\beta\)-galactosidase on nutrient and starvation agar (Lac\(^{+}\)). Presumably these transductants correspond to the construction illustrated in Fig. 8(b). With regard to germination, both the Lac\(^{+}\) and the Lac\(^{-}\) types showed the same defective behaviour as strain MR307.

Production of \(\beta\)-galactosidase by each class of transductants was measured throughout the *M. xanthus* life cycle. Those transductants classified as Lac\(^{-}\) on plates containing X-Gal proved to be so when expression of the lacZ gene was measured (see B1 fusion in Fig. 9). Fig. 9 also presents the profiles of \(\beta\)-galactosidase activity obtained for B2 fusion, a representative of the Lac\(^{+}\) phenotype. During vegetative growth, enzyme activity increased gradually from 6 units at early exponential growth to 45 units when the stationary phase was reached (Fig. 9a). Production of \(\beta\)-galactosidase during development is illustrated in Fig. 9(b). The level of enzyme activity for samples taken at \(t = 0\) was comparable to that found for late-exponential growth, when cells were spotted on CF agar plates. Enzyme production increased over vegetative levels during the first few hours, peaked at 24 h of development and then started decreasing, being particularly low late in development. The maximum level of enzyme production was reached at a time when microscopical observation revealed a majority of cells with spore morphology. \(\beta\)-Galactosidase activity during germination remained as low as for samples taken late in development (Fig. 9c).

The above results support the idea that there is a developmentally regulated promoter in or upstream of fragment B. The fact that all the Km\(^{R}\) transductants tested, regardless of their Lac phenotype, showed
Fig. 9. Expression of \(\beta\)-galactosidase in B1 (□), B2 (■), and C1 (▲) fusions during vegetative growth (a), development (b) and germination (c).

Germination-defective behaviour is consistent with the location of the promoter upstream of fragment B.

Since fragment C is located upstream of fragment B, we next measured expression of gene lacZ when fragment C is inserted before the reporter gene. Identification of a chimeric plasmid carrying insert C in the correct orientation with respect to the lacZ gene was possible by double restriction with SalI/XhoI. Such a chimeric plasmid was transduced into DK1050 with selection for Km\(^{R}\) (see Fig. 10), and the production of \(\beta\)-galactosidase and germination were assayed. The profiles of enzyme activity during cell growth, development and germination were very similar to those already presented for transductants carrying a tandem duplication of fragment B (see C1 fusion in Fig. 9). This result suggests that expression of lacZ in B fusions is dependent on a promoter located in fragment C. Several transductants tested showed a wild-type germination behaviour, indicating that in the construction shown in Fig. 10 a complete gerC allele must have been restored. Taken together these data suggest that the promoter of gene gerC is located in fragment C and that the mutant phenotype of strain MR307 results from disruption of a gene differentially expressed during development. Since MR307 undergoes normal development, the affected gene might encode a structural spore component unnecessary for development but essential early after induction of germination. There is an obvious link between development and germination. In particular, the early events during germination are very likely to depend on characteristics gradually acquired during sporulation. We have already mentioned that MR307 is blocked early during germination, and this is consistent with the fact that the gene is developmentally regulated.

Molecular analysis of gerC and its gene products will provide information about the specific defect that causes this mutant phenotype. On the other hand, the lacZ reporter gene should provide an easy way to search for trans-acting regulatory elements controlling gerC and perhaps also other genes involved in germination.

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References


