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# Differential Expression of Protein S Genes During *Myxococcus xanthus* Development

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**Protein S, the most abundant protein synthesized during development of the fruiting bacterium *Myxococcus xanthus*, is coded by two highly homologous genes called protein S gene 1 (*ops*) and protein S gene 2 (*tps*). The expression of these genes was studied with fusions of the protein S genes to the *lacZ* gene of *Escherichia coli*. The gene fusions were constructed so that expression of β-galactosidase activity was dependent on protein S gene regulatory sequences. Both the gene 1-*lacZ* fusion and the gene 2-*lacZ* fusion were expressed exclusively during fruiting body formation (development) in *M. xanthus*. However, distinct patterns of induction of fusion protein activity were observed for the two genes. Gene 2 fusion activity was detected early during development on an agar surface and could also be observed during nutritional downshift in dispersed liquid culture. Gene 1 fusion activity was not detected until much later in development and was not observed after downshift in liquid culture. The time of induction of gene 1 fusion activity was correlated with the onset of sporulation, and most of the activity was spore associated. This gene fusion was expressed during glycerol-induced sporulation when gene 2 fusion activity could not be detected. The protein S genes appear to be members of distinct regulatory classes of developmental genes in *M. xanthus*.**

*Myxococcus xanthus* is a gram-negative, gliding bacterium which exhibits a complex multicellular life cycle (for reviews, see references 19 and 33). During the vegetative phase, large numbers of rod-shaped cells move as "hunting groups," preying on other microorganisms or utilizing organic substrates in their path. Upon entering the developmental phase of its life cycle, *M. xanthus* forms multicellular structures called fruiting bodies, and individual cells become spherical or ovoid myxospores. Fruiting body formation depends on a number of factors, including nutritional deprivation, the availability of a solid surface, and cell density. The life cycle is completed with the provision of nutrients and the subsequent germination of the myxospores.

Dworkin and Gibson (10) have discovered that sporulation can also be induced in *M. xanthus* by the addition of high levels of glycerol (0.5 M) to cells growing vegetatively in liquid culture. This process, unlike fruiting body formation, occurs in growth media in the absence of a solid surface and is independent of cell density. In addition, it occurs very rapidly (2 to 4 h) as compared with the 2 to 3 days required for the production of fruiting body myxospores. Glycerol spores differ from fruiting body spores in several respects (32), but they also share certain properties, including resistance to physical breakage, heat, and desiccation. The synthesis of several proteins is induced during glycerol sporulation; at least one of these proteins, protein U, is also produced during fruiting body formation (21). The mechanism by which glycerol exerts its effect is unknown.

We have been interested in the mechanisms controlling the formation of fruiting bodies. One mechanism appears to be the expression of certain genes only during fruiting body formation (development); the products of these genes are assumed to play a role in the formation of the structures unique to *M. xanthus* development, multicellular aggregates and myxospores. Protein S and myxobacterial hemagglutinin (MBHA) are examples of proteins synthesized only during development. Protein S is an abundant protein ($M_r$, 19,000) found on the outer surface of myxospores which is synthesized from stable mRNA (9, 13–16, 29, 30). MBHA is a lectin-like protein ($M_r$, 28,000) which is produced at the time of cellular aggregation and is localized in the periplasmic space and at the cell poles of developmental, rod-shaped cells (6–8, 28). Neither of these proteins is produced in significant amounts during glycerol sporulation (6, 21).

The DNA encoding protein S was cloned in *Escherichia coli* to study its expression in detail. Two tandemly repeated protein S genes have been identified which are separated by about 1.4 kilobases (kb) on the *M. xanthus* chromosome (17). DNA sequencing has shown that the two genes are about 90% homologous (15). However, analysis of protein S and protein S RNA expression in *M. xanthus* strains with Tn*5* insertions in either protein S gene 1 or 2 (9) and the comparison of the partial amino acid sequence of protein S with the DNA sequences of the protein S genes indicates that only gene 2 encodes significant amounts of protein S (15). Protein S gene 1 expression has been detected with a gene 1-*lacZ* fusion (9). This expression, like the expression of protein S gene 2, is under developmental control in *M. xanthus*.

In this report, we extend our previous study of protein S gene regulation by comparing the expression of the previously described protein S gene 1 (*ops*; see reference 20) fusion with the corresponding protein S gene 2 (*tps*) fusion. In this investigation significant differences in the regulation of the two protein S genes were identified, and a basis for the definition of regulatory classes of developmental genes in *M. xanthus* has been suggested.

## MATERIALS AND METHODS

**Plasmids.** The plasmid vector for construction of the protein S gene-*lacZ* fusions, pMLB1034, has been described previously (3). pJDK5 (9) contains a 6-kb *Eco*RI-*Pst*I frag-

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ment of *M. xanthus* DNA which includes most of the protein S gene region of the *M. xanthus* chromosome (see Fig. 1). The plasmid pJMR100 carries most of the gene for the myxobacterial hemagglutinin (J. Romeo and D. Zusman, unpublished data).

The construction of the gene 1-*lacZ* fusion plasmid, pJDK8, and its Tn*5* insertion derivative, pJDK8-51, has been described previously (9). pJDK8 consists of a 1.6-kb *Sau*3A fragment from the gene 1 region of the *M. xanthus* chromosome inserted into the *Bam*HI site of pMLB1034 (Fig. 1). To provide a selectable kanamycin resistance (Kan$^r$) marker for transduction of the gene 1-*lacZ* fusion into *M. xanthus*, a plasmid (pJDK8-51) with a Tn*5* insertion in the *lacY* region of pJDK8 was isolated.

The gene 2-*lacZ* fusion plasmid, pJDK10, was constructed in a similar manner. From the DNA sequence of gene 2 (15) we observed a *Sau*3A site which corresponded in position (+52 to 55 base pairs) to the *Sau*3A site in gene 1 which was used to construct the gene 1-*lacZ* fusion. By using this site, a 1.04-kb *Sau*3A fragment from pJDK5 was purified by preparative polyacrylamide gel electrophoresis (25). This *Sau*3A DNA fragment contains the 5′ portion of gene 2 and an additional 1 kb of upstream *M. xanthus* DNA (Fig. 1). Ligation of this purified fragment to alkaline phosphatase-treated *Bam*HI-digested pMLB1034 was performed as described by Maniatis et al. (24). After transformation into *E. coli* MC1000 [F$^-$ *araD*139 Δ(*araABOIC-leu*)7679 *galUK* Δ(*lac*) × 74 *rpsL thi*] (3) plasmid DNA was isolated from ampicillin-resistant (Amp$^r$) transformants by the method of Birnboim and Doly (4) and screened by agarose gel electrophoresis of restriction endonuclease-cleaved DNA. pJDK10 contained the 1.04-kb *Sau*3A fragment in the correct orientation to generate an in frame fusion of the *M. xanthus* DNA encoding the 18 N-terminal amino acids of gene 2 with the *lacZ* gene. A Tn*5* insertion derivative of pJDK10 was isolated by the procedure described earlier (9). The Tn*5* insertion in this plasmid, pJDK10-51, is located in the Amp$^r$ genes of pJDK10 and provides a Kan$^r$ marker for transduction of the plasmid into *M. xanthus*. *E. coli* MC1000, containing pJDK10 or pJDK10-51, was found to be Lac$^-$ as indicated by the absence of a color reaction on LB X-gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) (Sigma Chemical Co.) plates.

***M. xanthus* protein S-*lacZ* fusion strains.** Plasmid DNA in *E. coli* MC1000 was transferred to *M. xanthus* DZF1 (27) by bacteriophage P1-mediated transduction, as described previously (31). Because pBR322-derived plasmids do not replicate in *M. xanthus*, Kan$^r$ *M. xanthus* transductants generally arise by the integration of one or more copies of the transferred plasmid into the bacterial chromosome by homologous recombination. Several independent pJDK8-51 and pJDK10-51 transductants were screened by Southern filter hybridization analysis of restriction endonuclease-cleaved genomic DNA to identify strains containing single copies of pJDK8-51 (DZF3361) and pJDK10-51 (DZF3427).

***M. xanthus* culture conditions.** The conditions for *M. xanthus* fruiting body formation on clone fruiting (CF) agar (11) plates have been described previously (29). To subject cells to nutritional downshift in liquid culture, cells growing vegetatively on Casitone-yeast extract (CYE) broth (5) were collected by centrifugation (8,000 × *g*), suspended in liquid CF medium at 2 × $10^8$ cells per ml, and incubated at 28°C with vigorous agitation. CF medium consisted of 10 mM Tris (pH 7.6)–1 mM K- or $NaPO_4$–8 mM $MgSO_4$–0.02% $(NH_4)_2SO_4$–0.2% sodium citrate–0.1% sodium pyruvate–0.015% Bacto Casitone (11). Glycerol sporulation was induced similarly, except that the vegetative cells were suspended in CYE broth–0.5 M glycerol as has been described previously (10).

**β-Galactosidase assays.** Cells were collected for β-galactosidase assays by suspending bacteria from one CF agar plate in 10 ml of TM buffer (10 mM Tris [pH 7.6], 10 mM $MgSO_4$) or by removing 10-ml samples of bacteria in liquid culture. The cells were pelleted by centrifugation at 10,000 × *g* and stored at −20°C. To determine the level of β-galactosidase activity, the cell pellets were suspended in 1 ml of Z buffer (0.1 M $NaPO_4$, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol [pH 7.0]) and sonicated for 2 min. The sonicated extracts were assayed as described previously (26). The protein concentration of the sonicated extracts was determined by the method of Lowry et al. (23).

**Southern filter hybridizations.** *M. xanthus* chromosomal DNA was prepared as described by Avery and Kaiser (2). The DNA was digested with restriction endonucleases (Bethesda Research Laboratories, Inc.) as described by the supplier. The transfer of DNA from agarose 0.7% gels to nitrocellulose filters (Sartorius), hybridization of DNA blots with $^{32}$P-labeled pJDK5, and the washing of the filters have been described previously (24). Radioactively labeled probe DNA was prepared by nick translation (24) with [α-$^{32}$P]dCTP (800 Ci/mmol; Amersham Corp.).

**Northern filter hybridization.** *M. xanthus* DZF1 RNA was prepared from developmental cells harvested from CF agar plates or from cells subjected to nutritional downshift in liquid CF medium. RNA was purified from sodium dodecyl sulfate cell lysates by phenol extraction, as described previously (12). DNase digestion (RNase-free DNase, Bethesda Research Laboratories) was used to eliminate DNA contamination of the RNA preparations. The fractionation of glyoxylated RNA by 1.5% agarose gel electrophoresis, the transfer of RNA to a nylon filter (Pall Biodyne A), and detection of protein S or MBHA RNAs by hybridization with nick-translated probe DNA have been described previously (9).

**Spore counts and purification.** Spore counts were done by determining the number of optically refractile, sonication-resistant spores in a Petroff-Hausser chamber. Purification of spores was performed by sedimentation through sucrose step gradients, as described previously (22).

## RESULTS

**Construction of protein S-*lacZ* gene fusions.** The protein S genes of *M. xanthus* were fused to the *lacZ* gene of *E. coli* with the pBR322-derived plasmid, pMLB1034 (3). This plasmid carries a truncated *lacZ* gene that is missing the DNA encoding the eight amino-terminal amino acids of β-galactosidase and all of the upstream sequences necessary for expression of the intact gene. Expression of *lacZ* is then dependent on the fusing of the truncated gene with the amino terminus of another gene in the correct reading frame and on supplying appropriate signals (i.e., promoter, ribosome binding site, and initiation codon) for expression of the fused gene. Restriction enzyme cleavage sites have been positioned near the N terminus of the *lacZ* gene on pMLB1034 to facilitate insertion of foreign DNA and the creation of gene fusions.

The strategy we used to construct the gene fusions is shown in Fig. 1. From the DNA sequence of the highly homologous protein S genes (15), we identified *Sau*3A sites at corresponding positions in both genes which were suitable for joining, in frame, the protein S genes and the *lacZ* gene of *Bam*HI-cleaved pMLB1034. Specifically, the gene 1 fu-

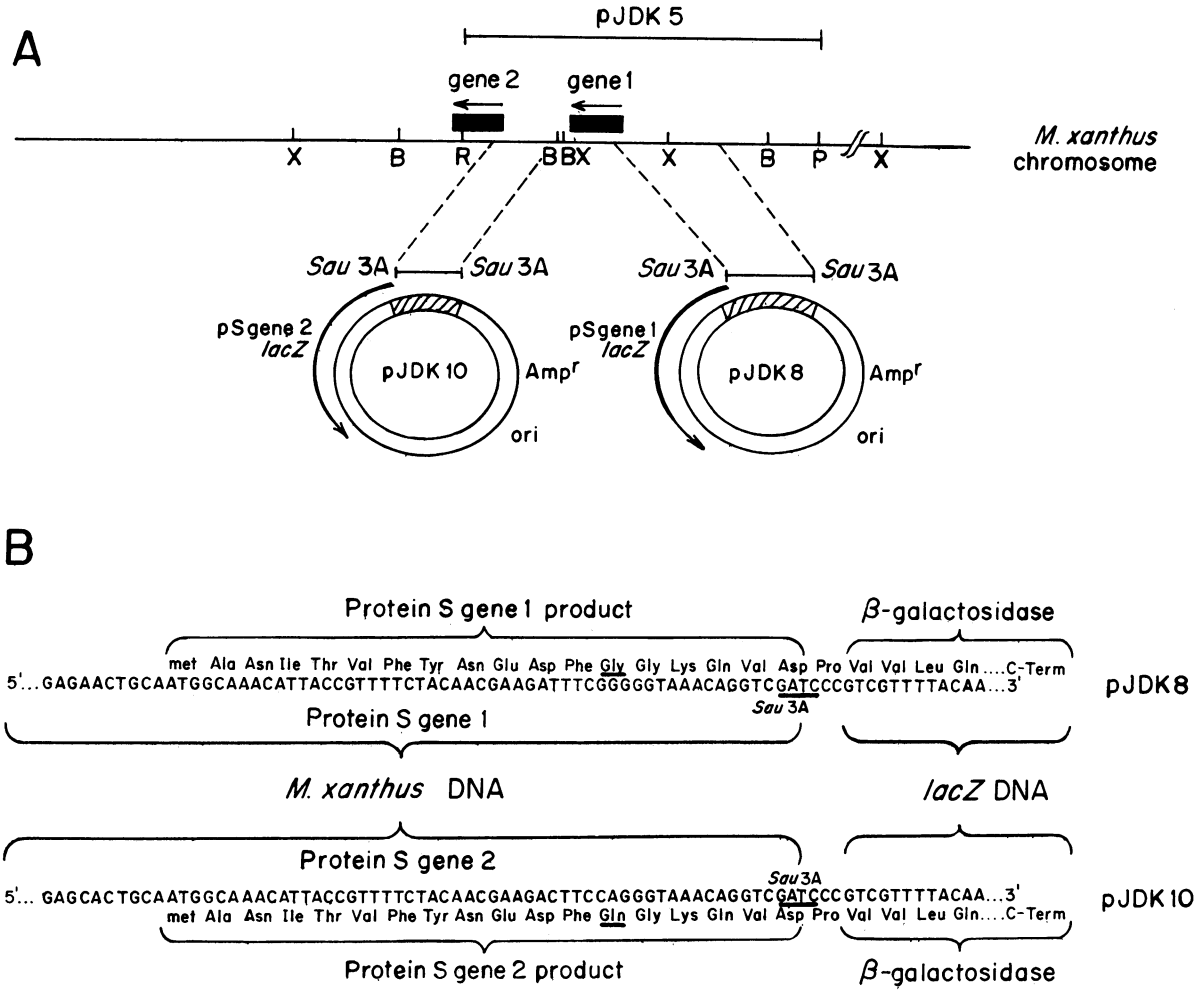


FIG. 1. Construction of protein S-*lacZ* gene fusions. (A) The region of the *M. xanthus* chromosome which contains the protein S genes is shown; the positions of the protein S genes (shaded boxes) and their orientation (arrows pointing 5′ to 3′) are indicated relative to the restriction map (B, *Bam*HI; P, *Pst*I; R, *Eco*RI; X, *Xho*I). *Sau*3A fragments from the gene 1 and gene 2 regions of the *M. xanthus* chromosome, 1.6 and 1.04 kb, respectively, were inserted into the *Bam*HI site of pMLB1034 to form the gene 1-*lacZ* fusion plasmid pJDK8 and the gene 2-*lacZ* fusion plasmid pJDK10. The *M. xanthus* DNA contained within these plasmids is indicated (hatched boxes), and the direction of transcription and translation required to express the fusion genes is shown with arrows. Also shown is the 6.0-kb fragment of *M. xanthus* DNA carried in plasmid pJDK5 (9). (B) The DNA and protein sequences from pJDK8 and pJDK10 at the site of fusion of the protein S genes with *lacZ* is shown (3, 9, 15). The *Sau*3A sites used to join the protein S genes with the truncated *lacZ* gene of pMLB1034 are underlined. The 18 amino acids encoded by the protein S genes at the amino-termini of the fusion proteins are identical, except at position 13 (underlined) at which the gene 1 fusion protein has glycine and the gene 2 product has glutamine.

sion plasmid, pJDK8, contains a 1.6-kb *Sau*3A fragment from gene 1 inserted into the *Bam*HI site of pMLB1034. This fusion plasmid contains 55 base pairs of the gene 1 structural gene (encoding 18 amino acids) fused to the *lacZ* sequences and about 1.5 kb of *M. xanthus* DNA upstream from the protein S gene 1 coding sequences. The gene 2 fusion plasmid, pJDK10, was formed in a similar manner by the insertion of a 1.04-kb *Sau*3A fragment from the gene 2 region into the pMLB1034 *Bam*HI site (Fig. 1). This fusion plasmid also contains 55 base pairs of structural gene sequences from gene 2 fused to the *lacZ* gene. In pJDK10, about 1 kb of additional *M. xanthus* DNA is present upstream from the gene 2 coding sequences. The predicted primary structures of the two hybrid proteins differ only at amino acid 13 at which the gene 1-*lacZ* hybrid protein contains glycine and the gene 2-*lacZ* protein contains glutamine (Fig. 1B).

**Introduction of protein S-*lacZ* gene fusion plasmids into *M. xanthus*.** To provide a selectable Kan$^r$ marker for transfer of gene fusions from *E. coli* to *M. xanthus*, Tn*5* insertions were isolated in nonessential regions of the pJDK8 and pJDK10 DNA. pJDK8-51 was found to contain Tn*5* within or near a part of the *lacY* gene carried by the plasmid vector, and pJDK10-51 has Tn*5* inserted in the Amp$^r$ gene of the parental plasmid.

The gene fusion plasmids pJDK8-51 and pJDK10-51 were transduced into *M. xanthus* with bacteriophage P1 and were selected for kanamycin resistance. As described previously (31), pBR322-derived replicons do not replicate in *M. xanthus*, and stable transduction of these plasmids depends on the integration of the DNA into the *M. xanthus* chromosome by homologous recombination. The Kan$^r$ transductants were analyzed for their ability to express β-galactosidase activity on CF agar containing the chromogenic substrate X-gal. All of the Kan$^r$ transductants tested (15 transductants contained pJDK8-51 and 50 contained pJDK10-51) expressed β-galactosidase activity developmentally (i.e., blue colonies

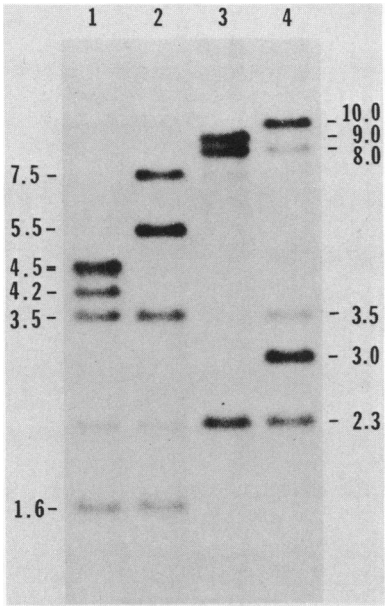


FIG. 2. Southern analysis of *M. xanthus* transductants containing the protein S-*lacZ* gene fusion. Restriction enzyme-cleaved chromosomal DNA from *M. xanthus* transductants containing protein S-*lacZ* gene fusions was analyzed by Southern filter hybridization. The hybridization probe was $^{32}$P-labeled pJDK5 which contains DNA from the protein S gene region of *M. xanthus* (Fig. 1) in the *E. coli* plasmid vector pUC8 (9). Hybridization patterns for DNA from the gene 1 fusion strain DZF3361 digested with *Xho*I and *Bam*HI are shown in lanes 1 and 3, respectively; whereas the patterns for *Xho*I- and *Bam*HI-cleaved DNA from the gene 2 fusion strain DZF3427 are shown in lanes 2 and 4, respectively. The sizes of the various DNA fragments identified in this analysis are indicated in kb based on molecular weight standards. The origin of these fragments is illustrated in Fig. 3.

formed on CF X-gal plates), but they did not produce detectable levels of activity while growing vegetatively (i.e., yellow colonies on CYE X-gal plates). These results indicate that all of the strains contain copies of the respective gene fusions and that both gene fusions are expressed in a developmentally regulated fashion. It is of interest that individual transductants of pJDK8-51 and pJDK10-51 differed with respect to the levels of β-galactosidase activity produced. Analysis of the pJDK10-51-containing transductants by Southern filter hybridizations indicated that the strains producing high levels of β-galactosidase activity contained multimeric units of the pJDK10-51 plasmid incorporated into the *M. xanthus* genome (data not shown). These results are consistent with the proposed mechanism for transduction of cloned DNA by bacteriophage P1 in which naturally occurring multimers of small plasmids are needed to fill the P1 heads (31). Upon entry of linear multimeric DNA into *M. xanthus*, two homologous recombination events are required to integrate the plasmid into the bacterial chromosome. Depending on the recombination sites involved, various numbers of the unit plasmid might be incorporated into *M. xanthus*.

Two transductants containing single copies of the fusion plasmids were identified for further study by Southern filter hybridization analysis: DZF3361, which contains pJDK8-51 (gene 1-*lacZ* fusion), and DZF3427, which contains pJDK10-51 (gene 2-*lacZ* fusion). The results on the analysis of the configuration of the transduced RNA in these strains are presented in Fig. 2. When *Xho*I-digested DNA from *M. xanthus* DZF3361 was hybridized with $^{32}$P-labeled pJDK5 DNA (Fig. 2, lane 1), three bands were observed (4.2, 3.5, and 1.6 kb) which were also observed in parental DZF1 DNA (data not shown). The 1.6- and 4.2-kb fragments contain protein S genes 1 and 2, respectively (Fig. 3). The 3.5-kb fragment is derived from DNA upstream of the protein S genes which is present in pJDK5. Two other bands of about 4.4 and 4.5 kb were also observed. These fragments are of the size expected for DNA extending from the *Xho*I sites located on either side of the pJDK8-51 gene fusion to the *Xho*I sites in the inverted repeats of Tn*5* (Fig. 3). When *Xho*I-digested DNA from *M. xanthus* DZF3427 was similarly analyzed (Fig. 2, lane 2), the 4.2-kb *Xho*I fragment containing protein S gene 2 was missing, as would be expected if pJKD10-51 had recombined into this region. Two larger *Xho*I fragments were observed with sizes (5.5 and 7.5 kb) expected for fragments extending from the *Xho*I sites outside the gene fusion plasmid to the *Xho*I sites in the ends of Tn*5* (Fig. 3). If DZF3427 contained multiple copies of the pJDK10-51 plasmid recombined into the chromosome, an additional band of about 8.4 kb would be expected, but it was not observed. Figure 2 (lanes 1 and 2) shows the presence of a faint band of about 2.3 kb. We think that this band originates outside the protein S gene region but that it weakly cross-hybridizes with the probe.

Figure 2 also shows the results of Southern hybridization analysis of *Bam*HI-digested DNA from DZF3361 and DZF3427. Three fragments from DZF3361 (9, 8, and 2.3 kb) hybridized strongly to the pJDK5 probe DNA (Fig. 2, lane 3). The 2.3-kb fragment was also found in *Bam*HI-digested DZF1 DNA and contains gene 2 (Fig. 3). A 3.0-kb fragment which contains protein S gene 1 was not observed, as would be expected if pJDK8-51 had recombined into this region of the *M. xanthus* chromosome. The 9- and 8-kb *Bam*HI fragments were of the expected sizes for *Bam*HI fragments composed of the 3.0-kb gene 1 fragment and the integrated pJDK-51. Another *Bam*HI fragment of about 13.7 kb would be predicted if multiple copies of pJDK8-51 were present. Both the 3.0-kb gene 1 fragment and the 2.3-kb gene 2 fragments were observed when DZF3427 DNA was analyzed (Fig. 2, lane 4). Two other *Bam*HI fragments (3.5 and 10 kb) were observed which had the predicted sizes for fragments composed of DNA from the *Bam*HI restriction sites flanking the gene fusion to the *Bam*HI site located in the center of Tn*5*. DNA from DZF3361 and DZF3427 was also digested with *Eco*RI, *Sal*I, and *Pst*I and analyzed by Southern hybridizations; the results were consistent with those described above.

In summary, the data presented above indicate that *M. xanthus* DZF3361 and DZF3427 have a single copy of the respective gene fusion plasmid incorporated into chromosomal DNA by homologous recombination between plasmid and genomic copies of *M. xanthus* DNA. In each strain, the gene fusion is preceded by the normal wild-type configuration of DNA upstream from the respective protein S gene. Each strain also contains unaltered copies of both protein S genes, although fusion plasmid sequences are present upstream from gene 1 in DZF3361 and gene 2 in DZF3427. It should be noted that both of the gene fusion strains appeared to be identical to DZF1 (parental strain) in the time course of development, the morphology of fruiting bodies, and the number of myxospores produced (data not shown).

**Expression of the protein S-*lacZ* gene fusions during fruiting body formation.** The kinetics of expression of β-galactosidase activity during fruiting body formation by *M. xanthus* DZF3361 (the gene 1-*lacZ* fusion strain) and DZF3427 (the

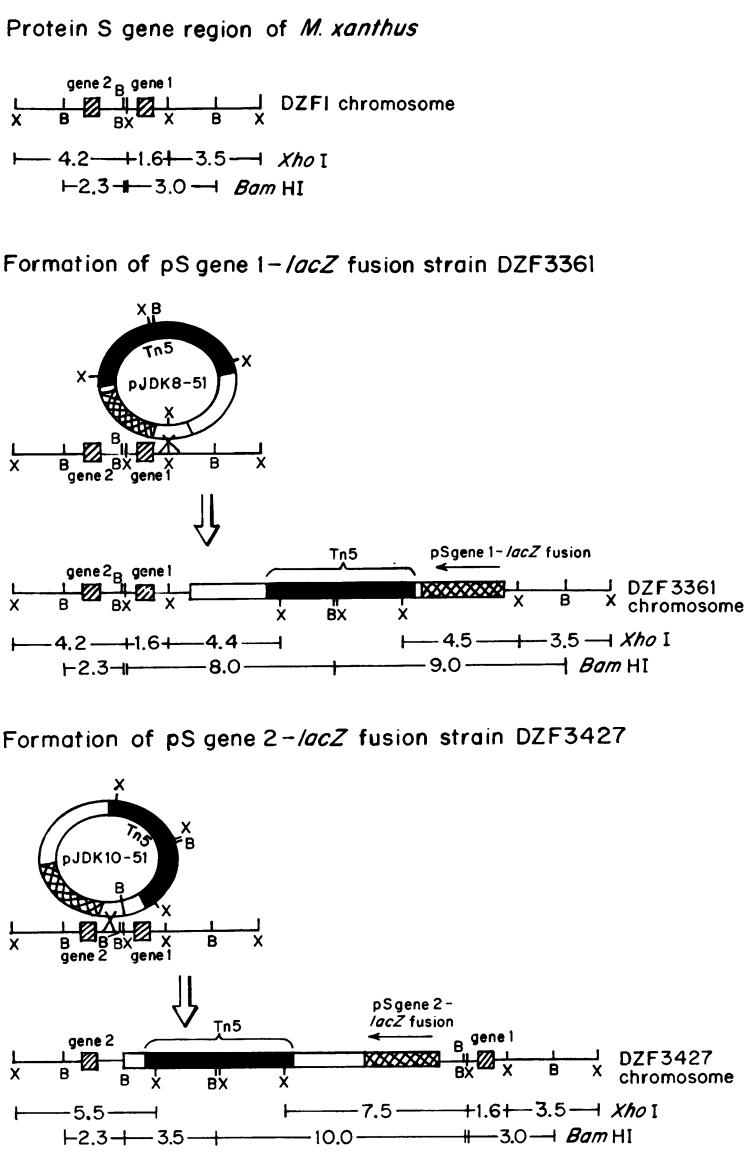


FIG. 3. Schematic representation of the orientation of the protein S gene fusions within the *M. xanthus* chromosome. (A) The protein S region of the *M. xanthus* DZF1 chromosome is shown with the positions of *Xho*I (X) and *Bam*HI (B) restriction sites and the sizes (in kb) of the *Xho*I and *Bam*HI DNA fragments. The protein S genes are indicated by hatched boxes. (B) The integration of the protein S (pS) gene 1-*lacZ* fusion plasmid pJDK8-51 into the DZF1 chromosome by homologous recombination resulted in the formation of the gene 1-*lacZ* fusion strain DZF3361, as shown. Tn*5* sequences are shaded, and gene 1-*lacZ* sequences are cross hatched. The direction of transcription of the gene fusion is indicated with an arrow. The *Xho*I and *Bam*HI fragments, shown in kb below the DZF3361 chromosome map, are those observed by Southern filter hybridization analysis of DNA from this strain (Fig. 2, lanes 1 and 3). The protein S genes are indicated by hatched boxes. (C) The integration of the protein S (pS) gene 2-*lacZ* fusion plasmid pJDK10-51 into the DZF1 chromosome by homologous recombination resulted in the formation of the gene 2-*lacZ* fusion strain DZF3427, as shown. Tn*5* sequences are shaded and gene 2-*lacZ* sequences are cross hatched. The direction of transcription of the gene fusion is indicated with an arrow. The *Xho*I and *Bam*HI fragments, shown in kb below the DZF3427 chromosome map, are those observed by Southern filter hybridization analysis of DNA from this strain (Fig. 2, lanes 2 and 4).

gene 2-*lacZ* fusion strain) are presented in Fig. 4. These experiments were performed by spotting concentrated cell suspensions on fruiting medium (CF agar) and removing samples of developing cells at various times during the course of several days of incubation at 28°C. Under these conditions, aggregation of cells into mounds occurred during the first 24 h, and sporulation occurred from 30 to 60 h. Developmental cell extracts were assayed for β-galactosidase activity. The time courses of expression of the two fusion proteins were markedly different (Fig. 4). A significant level of gene 1 fusion activity was not observed until 28 to 48 h of development. During this time interval, there was a major change in the cellular morphology of many cells, from long rod-shaped cells to short rods and optically refractile, ovoid myxospores. In contrast, the gene 2 fusion activity was clearly observed by 11 h. In some experiments, gene 2 activity was detected as early as 5 h of development (data not shown). The maximum level of gene 2-β-galactosidase expression was about 20-fold higher than the peak activity from gene 1. This observation is consistent with previous results which indicated that gene 2 is responsible for most of the expression of protein S. The time course of expression of the gene 2-β-galactosidase activity is also in accord with previous observations on the rate of protein S synthesis (13)

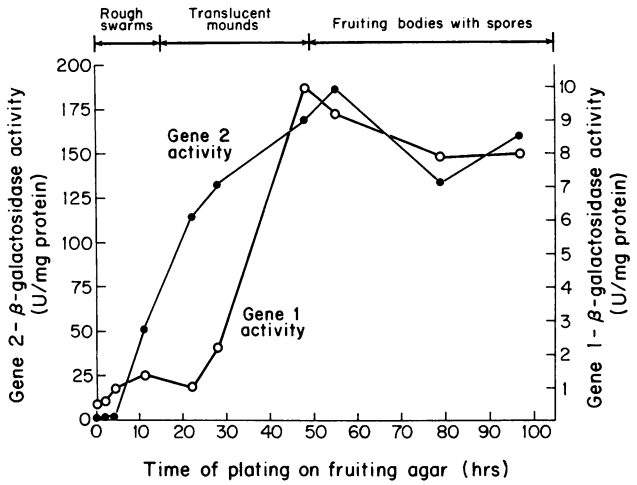


FIG. 4. Time course of protein S-*lacZ* induction during *M. xanthus* development. DZF3361 (gene 1-*lacZ* fusion strain) or DZF3427 (gene 2-*lacZ* fusion strain) cells were spotted onto CF agar plates at 28°C to initiate development. At the indicated times, developmental cells were harvested and assayed for β-galactosidase activity, as described in the text. The time course of the major fruiting events is indicated at the top of the figure. Sporulation occurs during the 28- to 48-h interval. Symbols: ○, gene 1 fusion activity; ●, gene 2 fusion activity.

and the relative amounts of protein S RNA found during development (9). These earlier studies have shown that protein S expression begins 3 to 6 h after cells have been plated on fruiting agar, peaks at about 20 h, and continues at a reduced rate later in development.

**Expression of the protein S-*lacZ* gene fusions during nutritional downshift in liquid culture.** *M. xanthus* fruiting body formation requires a solid surface and extensive intercellular contacts among large numbers of cells. To test whether these requirements also apply to the expression of the protein S genes, the *M. xanthus* gene fusion strains DZF3361 and DZF3427 were subjected to nutritional downshift in liquid shaker culture. For these experiments, cells were suspended in liquid CF medium at a cell density of $2 \times 10^8$ cells per ml, and the liquid cultures were incubated at 28°C with vigorous shaking. The downshifted bacteria were capable of only limited growth under these conditions, and the formation of extensive aggregates of cells and myxospores did not occur. Samples of cells from liquid CF culture were removed at various times for the determination of β-galactosidase activity. Only gene 2 was expressed in CF liquid shaker culture (Fig. 5). The activity was detected 6 h after the start of the experiment; by 17 h, a high level of activity was measured. The maximum specific activity observed in liquid culture, 550 U/mg of protein, was nearly three times the maximum level observed on developmental plates (Fig. 4). In contrast, the induction of the gene 1 fusion activity could not be detected in this experiment. These cells retained their ability to express gene 1 fusion activity under appropriate conditions because a portion of the DZF3361 liquid CF culture, removed at 42 h and spotted onto CF agar, produced a normal amount of the gene 1 fusion activity after an additional 48 h of incubation. At that time, the plated DZF3261 cells had aggregated and sporulated to form normal fruiting bodies.

**Expression of protein S RNA and myxobacterial hemagglutinin RNA during nutritional downshift in liquid culture.** Because the gene 2-*lacZ* fusion was expressed in liquid CF medium, it was of interest to determine whether protein S RNA was also produced under these conditions. RNA was therefore isolated from strain DZF1 after 20 h of incubation in CF liquid culture or after 20 h of development on CF agar and analyzed by Northern filter hybridization to $^{32}$P-labeled pJDK5. The results (Fig. 6A) indicate that protein S RNA is

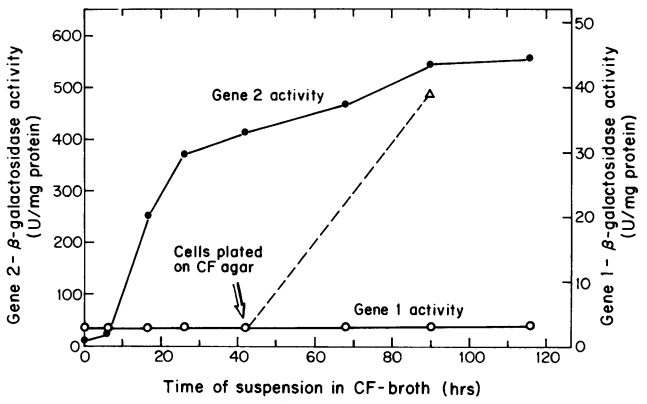


FIG. 5. Time course of protein S-*lacZ* induction during incubation of *M. xanthus* in liquid CF shaker culture. DZF3361 (gene 1-*lacZ* fusion strain) or DZF3427 (gene 2-*lacZ* fusion strain) cells were suspended in liquid CF medium at a cell density of $2 \times 10^8$ cells per ml. The cells were incubated at 28°C with vigorous shaking, and at the indicated times, 10-ml samples were removed and assayed for β-galactosidase activity as described in the text. In addition, a 20-ml sample of the DZF3361 culture was removed at 42 h, and after concentration by centrifugation, the cells were spread on a CF agar plate. The cells were harvested from this plate 48 h later and assayed for β-galactosidase activity (△). Fruiting bodies and spores had formed by this time on the agar surface. Symbols: ○, gene 1 fusion activity; ●, gene 2 activity.

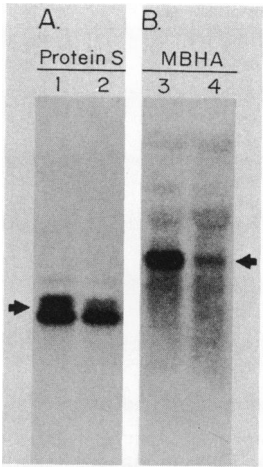


FIG. 6. Expression of protein S RNA and myxobacterial hemagglutinin RNA in liquid CF shaker culture. RNA was isolated from *M. xanthus* DZF1 after 20 h of development on CF agar plates or 20 h of incubation in liquid CF shaker culture. Protein S RNA or MBHA RNA was detected by Northern filter hybridization, as described in the text. (A) To detect the protein S RNAs, 10 μg of developmental (lane 1) or shaker culture RNA (lane 2) was hybridized to $^{32}$P-labeled pJDK5. (B) To identify MBHA RNA, 30 μg of developmental (lane 3) or shaker culture RNA (lane 4) was hybridized to $^{32}$P-labeled pJMR100. Arrows indicate the positions of the major protein S and MBHA RNA species.

present in cells from liquid culture at 20 h, although in a smaller amount than from developmental plate cultures at 20 h.

We also looked for expression of the myxobacterial hemagglutinin gene, because this gene is also known to be subject to developmental regulation in *M. xanthus* (6; J. Romeo and D. Zusman, unpublished data). The RNA samples described above were analyzed by Northern filter hybridization to a $^{32}$P-labeled pJMR100 probe; this plasmid contains most of the myxobacterial hemagglutinin gene (J. Romeo and D. Zusman, manuscript in preparation). The results (Fig. 6B) indicate that detectable amounts of the myxobacterial hemagglutinin RNA could also be found in cells incubated in the CF liquid culture. It should be noted that, as with protein S RNA, a smaller amount of myxobacterial hemagglutinin RNA was found at 20 h in liquid CF cultured cells than in developmental cells at 20 h.

**Expression of the protein S-*lacZ* gene fusion during glycerol-induced sporulation.** The addition of glycerol (0.5 M) to *M. xanthus* cells growing exponentially in liquid culture causes rod-shaped vegetative cells to convert to optically refractile spores in about 2 to 3 h (10); most, but not all, of these cells were sonication resistant. The effect of glycerol induction of

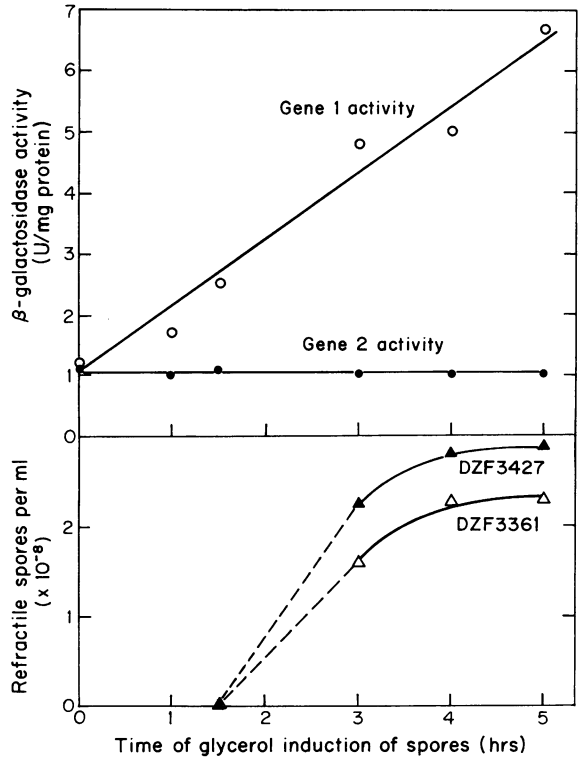


FIG. 7. Time course of protein S-*lacZ* induction during glycerol-induced sporulation. Glycerol-induced sporulation was initiated by suspending cells of the gene 1 fusion strain DZF3361 or gene 2 fusion strain DZF3427 in CYE broth containing 0.5 M glycerol at a cell density of $2 \times 10^8$ to $3 \times 10^8$ cells per ml. Samples (10 ml) of glycerol-induced bacteria were harvested at the indicated times and assayed for β-galactosidase activity, as described in the text. The number of sonication resistant, optically refractile glycerol spores was also determined with a Petroff-Hausser counting chamber. Symbols: ○, gene 1 fusion activity; ●, gene 2 fusion activity; △, DZF3361 spores; ▲, DZF3427 spores. The dashed lines indicate early times of glycerol-induced sporulation; this experiment does not indicate the earliest times that spores were observed.

sporulation on the expression of the protein S gene fusions in strains DZF3361 and DZF3427 was determined. Figure 7 shows that the gene 1 fusion activity was clearly observed early during glycerol induction, before the appearance of refractile spores. In addition, the gene 1 fusion activity continued to increase even after the morphological conversion of the cells was complete. In contrast, the expression of the gene 2 fusion could not be detected during glycerol-induced sporulation.

**Effect of temperature on protein S-*lacZ* gene fusion expression.** We noticed that temperature markedly affects the level of protein S gene fusion activity during development. To study this effect, the time course of expression of the protein S gene fusions was measured during development induced at 28, 34, and 37°C. The results of this experiment (Fig. 8) indicate that the specific activity of the gene 2 fusion declined significantly with an increase in temperature. This effect became more dramatic with increasing time of development (Fig. 8A). At 70 h, the gene 2 fusion activity at 28°C was found to be about 10-fold greater than that at 37°C. In this experiment, cells were plated at a low cell density ($7 \times 10^8$ cells per plate; about 1/10 the cell density routinely used for development); at higher cell densities ($7 \times 10^9$ cells per plate), similar results were obtained (data not shown).

A different response to temperature increase was observed for the expression of the gene 1 fusion activity. No gene 1 fusion activity was detected at 28°C, whereas relatively high levels of activity were detected at 34 and 37°C (Fig. 8C). As noted above, the appearance of gene 1 fusion activity was correlated with the production of optically refractile myxospores. At the low cell density used in this experiment, sporulation did not occur at 28°C. However, at higher temperatures, sporulation did occur, and gene 1 fusion activity could be detected. It is interesting that the gene 1 fusion specific activity and the total activity recovered per plate (data not shown) continued to increase after spore production had reached its maximal level. When cells were plated at a high cell density ($7 \times 10^9$ cells per plate), similar results were obtained (data not shown). The most notable differences observed were that, as expected, sporulation and expression of gene 1 fusion activity did occur at 28°C, as well as at 34 and 37°C, and that myxospores and gene 1 fusion activity were produced earlier than at a low cell density.

**Differential localization of protein S-*lacZ* gene fusion activities.** The correlation between the expression of the gene 1 fusion activity and the production of myxospores suggested that the gene 1 fusion activity might be spore associated. We therefore investigated the location of both of the protein S gene fusion activities during fruiting body formation. Developmental DZF3361 and DZF3427 cells were harvested from CF-agar plates at various times of development, disrupted by sonication, and separated into soluble and particulate fractions after low-speed centrifugation. The fraction of total β-galactosidase activity which remained soluble was then determined. The results (Fig. 9) indicate that although most (over 85%) of the gene 2 fusion activity was found in the soluble fraction throughout development, the gene 1 fusion activity was primarily found in the particulate fraction. For example, at 40 h of development, 90% of the gene 1 fusion activity was associated with the particulate fraction, whereas only 11% of the gene 2 activity was in that fraction. At later stages of development (i.e., 72 h), the fraction of gene 1 activity in the soluble fraction increased to 45%.

Myxospores can be purified from other material in the particulate fraction by sucrose gradient velocity centrifuga-

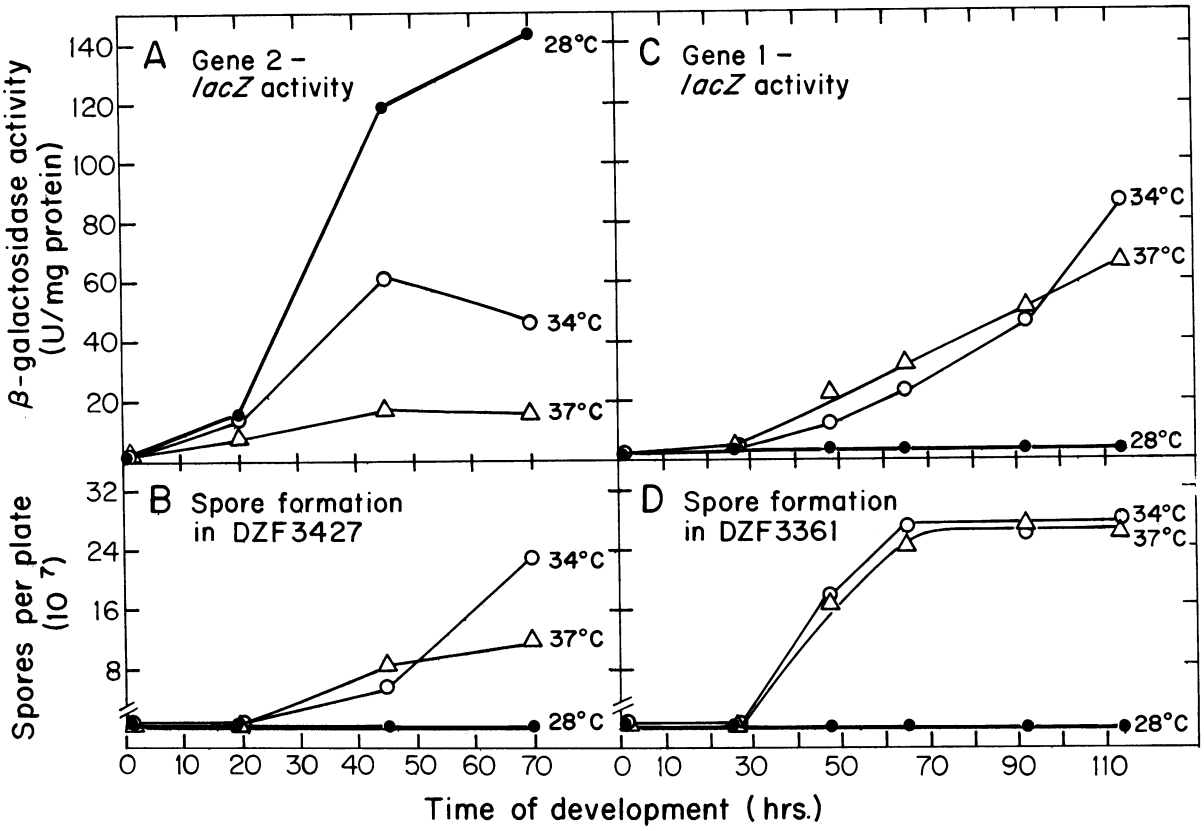


FIG. 8. Effect of temperature on protein S-*lacZ* expression during *M. xanthus* development. Development was initiated by spotting cells of the gene 1-*lacZ* fusion strain DZF3361 or the gene 2-*lacZ* fusion strain DZF3427 on CF agar plates at a cell density of $7 \times 10^8$ cells per plate. The plates were then incubated at 28, 34, or 37°C. At the indicated times, samples of developmental cells were harvested and assayed for β-galactosidase activity. The number of sonication-resistant, optically refractile spores was also determined with a Petroff-Hausser counting chamber. (A) Protein S gene 2-*lacZ* fusion activity. (B) DZF3427 spores formed per plate. (C) Protein S gene 1-*lacZ* fusion activity. (D) DZF3361 spores formed per plate. Symbols: ●, 28°C; ○, 34°C; △, 37°C.

tion. This treatment removes most of the cell debris, as well as a substance(s) from spores which alters their surface properties (22). In Table 1 is shown the data we obtained when the particulate fraction from developmental cells at 48 h was assayed for β-galactosidase activity before and after spore purification. The gene 1-*lacZ* fusion activity was clearly spore associated because the activity found in $10^8$ spores actually increased after spore purification. This result suggests that the purification procedure might remove some interfering substance from the spore preparations. Table 1 also shows that some of the gene 2-*lacZ* activity appears to be spore associated. However, this represents a relatively small portion of the total activity because only 15% of gene 2-*lacZ* fusion activity is found in the particulate fraction of developmental cells at 48 h (see Fig. 9).

## DISCUSSION

The fruiting myxobacterium *M. xanthus* contains two highly homologous protein S genes (9, 15, 17). Earlier studies have shown that gene 2 is responsible for most of the synthesis of protein S, but have also suggested that gene 1 is expressed during fruiting body formation (9). We have extended our study of the expression of the protein S genes by constructing gene fusions of gene 1 and gene 2 to the *lacZ* gene from *E. coli* to produce enzymatically active hybrid proteins. When these gene fusions were introduced into *M. xanthus*, expression of the fusion proteins was controlled by protein S DNA sequences upstream from the hybrid genes. The surprising conclusion from the studies reported here is that the protein S genes exhibited distinct patterns of expression, suggesting that they are members of different classes of developmentally controlled genes.

Protein S gene 2 (*tps*) is a highly expressed developmentally regulated gene. By measuring the β-galactosidase activity under the control of gene 2, we were able to show that the fusion protein exhibited approximately the same kinetics of synthesis as previously determined for protein S during development (13). Expression of the fusion gene was first observed about 4 h after initiation of development and continued at a high rate until about 20 to 30 h; at later times the activity decreased.

Interestingly, gene 2 was also found to be expressed during nutritional downshift of *M. xanthus* in liquid shaker culture. This conclusion is based on the detection of appreciable levels of gene 2 fusion activity and protein S RNA under these conditions. At the cell density used in these experiments ($\simeq 2 \times 10^8$ cells per ml), only very limited intercellular contacts were possible and sporulation did not

occur. Apparently, activation of gene 2 expression occurs primarily in response to nutritional limitation of *M. xanthus* cells. The MBHA gene was also expressed during liquid CF medium culture. MBHA is another abundantly produced developmental protein which begins to be synthesized early during fruiting body formation (7). MBHA RNA was detected at 20 h after nutritional downshift, as indicated by Northern filter hybridization analysis using the cloned MBHA gene as a probe.

As reported earlier, protein S gene 1 (*ops*) is expressed at relatively low levels in *M. xanthus*, and the protein and RNA products of this gene were not identified (9). However, with the gene 1-*lacZ* fusion, it was possible to show that expression of this gene does occur and that it is developmentally regulated. In general, the time of expression of gene 1 fusion activity was correlated with the time of spore production. This correlation was found both during fruiting body formation (Fig. 4) and during glycerol induction of spores (Fig. 7). Also, when cells were placed in CF liquid shaker culture, protein S gene 1 fusion activity was not detected and sporulation did not occur (Fig. 5). Another developmental protein, protein U, has a pattern of expression similar to that of gene 1. This protein is produced beginning at about 48 h of development (13) and during glycerol induction of sporulation (21). Also, it may be significant that, during glycerol sporulation, induction of gene 1 preceded spore formation. We expect that the expression of a number of genes is required for the formation of glycerol spores. If this is the case, then these sporulation genes may exhibit patterns of expression like those of gene 1.

It appears that the protein S genes are members of distinct regulatory classes of developmental genes. However, because very few developmental *M. xanthus* genes have been identified, the significance and importance of these classes of genes during *M. xanthus* development is currently unknown. With regard to these questions, this comparative study of the protein S genes provides an experimental framework for the classification of additional developmental genes as they are discovered. In addition, the determination of conditions which allow the selective expression of either gene 1 or gene 2 indicates that additional developmental genes with regulatory properties similar to those of the protein S genes may be identified by virtue of their expression during glycerol-induced sporulation or in liquid CF shaker culture.

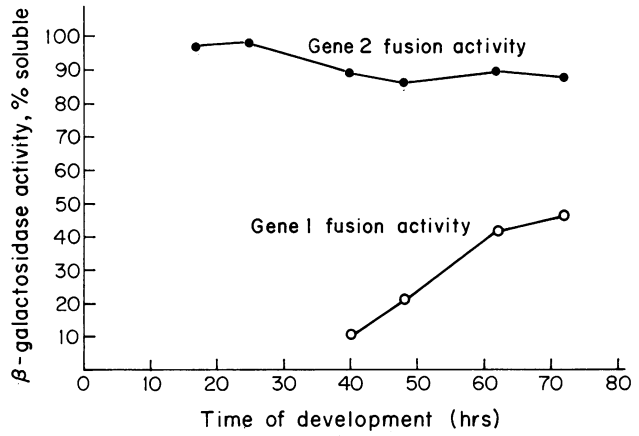


FIG. 9. Differential localization of the protein S gene fusion activity during *M. xanthus* development. Developmental DZF3361 (gene 1-*lacZ* fusion strain) or DZF3427 (gene 2-*lacZ* fusion strain) cells were harvested at the indicated times, and sonicated lysates were prepared. Particulate and soluble fractions of the lysates were assayed for β-galactosidase activity, and the fraction of the total activity that was found in the soluble fraction was determined. Symbols: ○, soluble gene 1 fusion activity; ●, soluble gene 2 fusion activity.

TABLE 1. Analysis of spores for protein S-*lacZ* fusion activity

| Strain | β-Galactosidase activity[a] | | |
|---|---|---|---|
| | Particulate fraction | Purified spores[b] | Recovery (%) |
| DZF3361 (contains gene 1-*lacZ* fusion) | 1.17 | 2.49 | 213 |
| DZF3427 (contains gene 2-*lacZ* fusion) | 2.28 | 1.53 | 68 |

[a] The particulate fraction of developmental cells at 48 h was assayed for β-galactosidase activity as described in the text. Activity is expressed as change in optical density at 420 nm per 30 min per $10^8$ spores.

[b] Spores were purified from the particulate fraction in a sucrose gradient, as described by Kupfer and Zusman (22). The purified spores were then assayed for β-galactosidase activity as described in footnote *a*.

Protein S gene 2 is expressed at a much higher level than gene 1 under the fruiting conditions generally used for *M. xanthus* development (i.e., high cell density on CF agar at 28 to 30°C). However, the relative level of expression from these genes is sensitive to the conditions of development. For example, gene 2 expression is greatly reduced at elevated temperatures such as 34 or 37°C (Fig. 8). (It has been known for several years that yields of protein S and myxobacterial hemagglutinin are much lower at 34°C than at 28°C; unpublished observations.) Protein S gene 1 fusion activity, on the other hand, was found to be at least as great at 34 and 37°C as at 28°C. The effect of temperature on protein S gene expression and sporulation was particularly interesting when cells were plated at low cell densities (Fig. 8). At 28°C, both sporulation and the expression of gene 1 fusion activity did not occur, whereas at 34 or 37°C, both were observed. These results provide another example of the correlation between the induction of gene 1 fusion activity and sporulation. They also suggest that temperature plays an important role in the control of sporulation in *M. xanthus*. Recent work in our laboratory shows that *M. xanthus* has a temperature-dependent pathway for aggregation (K. O'Connor and D. Zusman, manuscript in preparation). The effect of temperature on development of *M. xanthus* is complex, but the response to elevated temperatures might be similar in some respects to the heat shock response found in other bacteria or higher organisms (1), because 34 and 37°C are close to the maximum temperature for growth of this bacterium (18). It is possible that other types of stress might also be expected to influence the relative level of expression of the protein S genes and the decision of cells to sporulate.

In addition to differences in the pattern of expression of the protein S gene 1 and gene 2 fusions, we also observed a difference in the localization of the fusion proteins. Simple cell fractionation procedures were used to show that throughout development, most of the gene 2 fusion activity was in a soluble cell fraction, whereas the gene 1 fusion activity was in a particulate fraction (spore associated). This result is puzzling because the gene 1 and gene 2 fusion proteins are predicted (from DNA sequence comparisons) to have only a single amino acid difference, located at position 13 from the amino terminus. The significance of these findings is not understood at this time.

In summary, the use of protein S-*lacZ* gene fusions has allowed the detection of distinct patterns of regulation for

the expression of the two protein S genes of *M. xanthus*. Protein S gene 2 expression began early in development and appeared to respond primarily to nutritional deprivation of the bacteria. Now it will be possible to look more precisely at the nutritional factors involved in the induction of early developmental gene expression. Protein S gene 1 was expressed much later in development and was induced at about the same time that sporulation commenced. Analysis of glycerol-induced, synchronously sporulating cells showed that induction of gene 1 preceded the appearance of spores and suggests that gene 1 expression occurs immediately before sporulation during fruiting body formation. Regulation of gene 1 expression during fruiting body formation appears to be complex and probably depends on interactions between cells, interactions which are disrupted in shaker culture. These gene fusions should allow the identification of the protein S gene sequences that are necessary for their regulation and of other genes involved in the control of their expression. The fusion proteins will also be particularly useful as markers of developmental gene expression in the dissection of the complex, multicellular process of fruiting body formation.

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