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Localization of the *cis*-Acting Regulatory DNA Sequences of the *Myxococcus xanthus tps* and *ops* Genes

JOHN S. DOWNARD,* SANG-HOON KIM, AND KI-SOO KIL

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019

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The *cis*-acting regulatory regions of the *tps* and *ops* genes of *Myxococcus xanthus* were localized by analyzing the expression of fusions of these genes with the *lacZ* gene. A 201-base-pair (bp) fragment of *tps* DNA extending 95 bp upstream (−95) from the transcriptional start was sufficient to direct developmentally regulated expression of fusion gene activity. The segment of *tps* DNA between −95 and −81 contained information necessary for developmental regulation. A segment of *ops* DNA extending upstream to −131 directed a very low level of *ops-lacZ* fusion expression, but the inclusion of DNA to −208 greatly increased the amount of developmentally regulated expression. *M. xanthus* DNA upstream from −108 in the *tps* gene and −311 in the *ops* gene was required for maximal expression of gene fusion activity. The upstream regulatory regions of both the *tps* and *ops* genes seem to be involved in positive transcriptional regulation. Two mutations, a deletion of 1 bp at −8 in the *tps* gene and a 3-bp substitution at −27 to −29 in the *ops* gene, greatly increased the level of vegetative expression of gene fusion activity, suggesting that both genes may also be subject to negative regulation in *M. xanthus*.

Myxococcus xanthus is a gram-negative, gliding bacterium with a complex life cycle involving groups of cells (14, 27). Vegetative cell groups prey on other microorganisms or utilize organic nutrients in their environment (primarily soil). Starvation of these groups of cells results in their aggregation into mounds (fruiting bodies) and the differentiation of individual rod-shaped bacteria into spherical or ovoid myxospores within the fruiting bodies. This dramatic response to nutrient depletion is referred to as development. Mature fruiting bodies, which require 2 to 3 days to form, may contain 10^6 spores. Sporulation can also be relatively rapidly induced (3 to 5 h) in vegetatively growing cells by the addition of glycerol (0.5 M); aggregation is not required (6). However, glycerol-induced spores differ markedly from developmental spores in ultrastructure (9).

One of the mechanisms controlling development in *M. xanthus* appears to be the regulation of gene expression (13). Our studies have focused on attempting to identify the molecular mechanisms controlling the expression of the *tps* and *ops* genes (formerly protein S genes 2 and 1, respectively). These genes are about 90% homologous at the DNA sequence level and are separated by 1.4 kilobase pairs (kbp) on the *M. xanthus* chromosome (11, 12). The expression of these genes has been studied by analysis of *tps* and *ops* RNA and by assay of β-galactosidase activity directed by translational fusions of these genes to the *Escherichia coli lacZ* gene (2, 4, 5). These studies have indicated that the *tps* and *ops* genes are differentially expressed, with *tps* expression beginning about 5 h after the initiation of development and before aggregation has occurred, and *ops* expression beginning at about 40 h as spores are forming within fruiting bodies. The *tps* gene is expressed in starvation shaker culture in the absence of aggregation, while the *ops* gene is not expressed under these conditions. In contrast, the *ops* gene, and not the *tps* gene, is expressed during glycerol-induced sporulation. The expression of both genes is apparently controlled at the transcriptional level (2, 4). The functions of the protein products of the *tps* and *ops* genes are unknown.

Currently, little is known about genes which control the expression of the *tps* and *ops* genes. However, it has been shown recently that *tps* and *ops* gene expression is controlled by SpoA and SpoB (*ssbA*) loci (7, 15). Mutations at these loci block sporulation and expression of the *tps* and *ops* genes as well as a number of other developmentally regulated genes. Sporulation and developmental gene expression can be rescued by mixing these mutant strains with a wild-type strain. These and other observations suggest that the spo mutant strains are defective in the production of signal molecules which must be passed between cells for developmental gene expression and sporulation to occur normally. However, if cells which can produce the signals are present, the spo strains are capable of signal reception and will develop normally (8, 16). Four groups of spo mutants have been identified on the basis of the ability of members of different groups to stimulate each other to sporulate. These results suggest that at least four signals exist which have specific effects on developmental gene expression in *M. xanthus*. The *tps* and *ops* genes, as targets of this control, are attractive subjects for studies of the signal transduction circuits involved in the regulation of developmental gene expression.

Little is known as well about the *cis*-acting *tps* and *ops* DNA sequences involved in controlling gene expression. The DNA sequences of the two genes are greater than 90% related for about 100 base pairs (bp) upstream from the translational initiation codon (11). Homology between the genes ends abruptly at that point. The 5′ ends of both *tps* and *ops* RNA map 50 bp upstream from the translational start (2, 10), suggesting that transcription initiates at corresponding positions in the two genes. An interesting feature of this system is that comparison of the DNA sequences in the regulatory regions of the *tps* and *ops* genes may be helpful in developing an understanding of how differential regulation is achieved and how the regulatory systems which control gene expression may be related.

With the goal of understanding the molecular mechanisms

* Corresponding author.

controlling developmental gene expression in *M. xanthus* and identifying the regulatory proteins involved, we have localized the regulatory regions of the *tps* and *ops* genes. The regulatory regions of the two genes were found to include DNA sequences upstream from the presumptive transcription initiation sites in both genes, including areas in which the *tps* and *ops* genes are related and regions farther upstream in which they differ extensively.

MATERIALS AND METHODS

***M. xanthus* culture conditions.** *M. xanthus* cultures were grown vegetatively in Casitone-yeast extract (CYE) medium (1), and glycerol-induced sporulation was carried out in the same medium containing 0.5 M glycerol at 30°C as has been described elsewhere (5). Development in liquid shaker culture was initiated by suspending vegetatively grown cells, which had been collected by centrifugation, in clone-fruiting (CF) medium at a density of 2×10^8 to 4×10^8 cells per ml (5). Under these conditions the *M. xanthus* cells appear to pass through the early developmental stages (including expression of the *tps* gene) but do not aggregate or sporulate. The conditions for development on CF agar plates have been described elsewhere (21).

Plasmids. The *lacZ* translational fusion vector pMLB1034 was used in all the gene fusions described in this paper (25). The gene fusion plasmids contained *M. xanthus* DNA (Fig. 1). In all the gene fusion plasmids the *tps* or *ops* DNA sequences are joined to the *lacZ* DNA at a *Sau*3A site, found at the corresponding position within both genes, so that the N-terminal 18 amino acids of the *tps* and *ops* protein products are at the N terminus of each fusion protein (4, 5). The plasmids pJDK26 and pSK3 were constructed by inserting the 201-bp *Hin*fI-*Sau*3A or 214-bp *Eco*O109-*Sau*3A *tps* DNA fragments, respectively, into the *Bam*HI-*Sma*I-cleaved pMLB1034. Blunt ends were formed at the *Hin*fI and *Eco*O109 ends of these fragments by the fill-in reaction by using the Klenow fragment of DNA polymerase I (17). The restriction fragments were purified by polyacrylamide gel electrophoresis before ligation to the vector (18). Insertions of transposon Tn*5* into the ampicillin resistance gene of the resulting plasmids were identified as described earlier (4). A series of deletion plasmids (pSK1 series) was constructed by *Bal* 31 (New England BioLabs, Inc.) treatment of pJDK26 which had been previously digested with *Eco*RI. The unique *Eco*RI site in pJDK26 is immediately upstream from the *M. xanthus* sequences in this plasmid. The *Bal* 31-digested plasmid DNA was recircularized by ligation in the presence of *Xba*I linkers (New England BioLabs). After transformation of *E. coli*, plasmids which had lost the *Eco*RI site and gained an *Xba*I site were identified. The extent of the deletion was characterized by agarose gel electrophoresis of restriction enzyme-cleaved DNA and then by chain termination DNA sequencing (24) (New England BioLabs kit) of M13mp18 phage (22) containing *Xba*I-*Pvu*II fragments with the *tps* DNA segments from the various plasmids.

The *ops-lacZ* fusion plasmids are derivatives of the *ops-lacZ* plasmid pJDK8, which has been described previously (4, 5). The *Hin*dIII-*Eco*RI fragment from pJDK8 was removed and replaced with the polylinker region from M13mp18 to create pKS7. Three plasmids, pKS8, pKS9, and pJDK55, were constructed by inserting the 124-bp *Pvu*II-*Hin*dIII, the 200-bp *Xmn*I (filled-in)-*Hin*dIII, and the 303-bp *Nci*I (filled-in)-*Hin*dIII *ops* fragments, respectively, into pKS7 which had been cleaved with *Sma*I and *Hin*dIII. These manipulations resulted in *ops-lacZ* fusions with unaltered

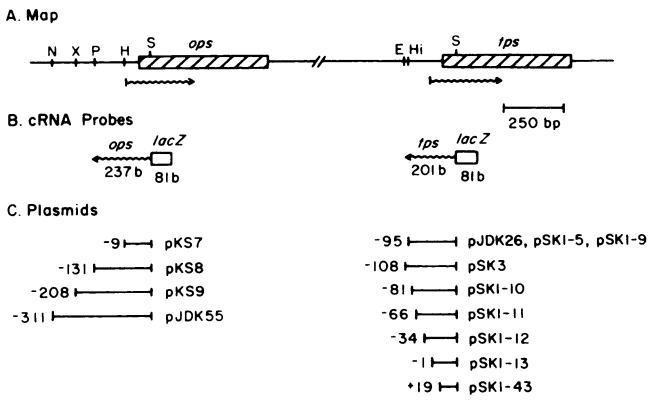

FIG. 1. *M. xanthus* DNA present in gene fusion plasmids and cRNA probes used for analysis of fusion gene expression. (A) Map of the *tps* and *ops* genes on the *M. xanthus* chromosome is shown with the locations of selected restriction endonuclease sites. The protein-coding regions of the two genes (▨) and the direction of RNA synthesis from the transcription initiation sites (⟿) are indicated. About 1.4 kb of DNA separates the *ops* and *tps* genes on the chromosome. Restriction sites: N, *Nci*I; X, *Xma*I; P, *Pvu*II; H, *Hin*dIII; S, *Sau*3A; E, *Eco*O109; Hi, *Hin*fI. (B) cRNA probes were synthesized as described in Materials and Methods. The *ops-lacZ* probe consisted of 81 bases from *lacZ* (5′ end) and 237 bases from the *ops* gene (*Sau*3A site to *Pvu*II site). The *tps-lacZ* probe consisted of the same 81 bases from *lacZ* and 201 bases from the *tps* gene (*Sau*3A site to *Hin*fI site). These probes are precisely complementary to the 5′ ends of correctly initiated RNA from the *ops-lacZ* and *tps-lacZ* fusions. (C) The segments of *M. xanthus* DNA from the gene fusions described in this study are shown (bars). The right-hand border of each segment is the *Sau*3A site from either the *ops* or the *tps* gene shown on the map above. The number at the left-hand border indicates the upstream end of the DNA segment relative to the putative transcriptional start for each gene. The start sites for both genes are 50 bp upstream from the translational initiation codons. Gene fusions including these *M. xanthus* DNA segments are included on the plasmids indicated to the right of the bars.

ops DNA sequences extending 9 bp (pKS7), 131 bp (pKS8), 208 bp (pKS9), and 311 bp (pJDK55) upstream from the transcriptional start. For transduction to *M. xanthus*, all these plasmids contained Tn*5* insertions in the ampicillin resistance gene of pMLB1034.

The template plasmid for the synthesis of the *tps-lacZ* cRNA probe, pJDK30, was constructed by inserting the 289-bp *Eco*RI-*Pvu*II fragment from pJDK26 into the vector plasmid, pT7/T3-19 (Bethesda Research Laboratories, Inc.). The inserted DNA fragment contains all of the *M. xanthus* DNA (201 bp) and the first 81 bp of the β-galactosidase coding sequences from pJDK26 and is positioned so that antisense RNA (cRNA) could be synthesized from a bacteriophage T7 promoter located just outside the *lacZ* end of the insert. The plasmid pJDK35 served as the template for the synthesis of the *ops-lacZ* cRNA probe and was constructed by inserting the 318-bp *Pvu*II fragment from pJDK8 into the vector plasmid pGEM-3 blue (Promega Biotec). This *Pvu*II fragment also contained 81 bp of *lacZ* DNA along with 237 bp of *ops* DNA, and, again, the fragment was oriented so that antisense RNA could be produced from a T7 promoter.

Oligonucleotide site-directed mutagenesis. Mutations were introduced into the *tps* and *ops* DNA by using synthetic oligonucleotides incorporating the desired base changes. For this purpose the *Hin*fI (filled-in)-*Sau*3A fragment from the *tps* gene (the same fragment used to make pJDK26) was inserted into M13mp19 which had been cleaved with *Bam*HI

and *Sma*I, and the *Eco*RI-*Hin*dIII fragment from pJDK55 (containing *ops* DNA to position −311) was inserted into M13mp18. A kit from Amersham, Inc., based on the procedure of Taylor et al. (26) was used for site-directed mutagenesis and enrichment for M13 phage containing mutant DNA sequences. Alterations in the DNA sequence were confirmed by DNA sequence analysis (24). The change introduced into the *tps* DNA sequence was the deletion of a T residue at position −11 (which creates a *Hin*dIII site) (designated D1; see Fig. 6). The change in the *ops* DNA was the substitution of the sequence ATT for TCC at positions −27 to −29 (D3). The mutagenic oligonucleotides were 5′-GAGCGCGGTGCCCAAGCTTCCGGCGGCTTC-3′ (D1) and 5′-AGGCGGATTGCATTTCCGGAGCGCG-3′ (D3). These were synthesized on an Applied Biosystems DNA synthesizer in the laboratory of B. Roe (University of Oklahoma). The mutant *tps* DNA segment was used to construct *tps-lacZ* fusions as was described for the construction of pJDK26. The mutant *ops* DNA segment was used to construct an *ops-lacZ* fusion as was described for pJDK55.

Bacteriophage P1 transduction. Plasmid DNA sequences containing gene fusions were transferred for *E. coli* to *M. xanthus* DZF1 by P1 transduction essentially as has been described (23). The recipient DZF1 (*M. xanthus* wild type) cells were concentrated to approximately 4×10^9 cells per ml in CYE medium before phage infection, and occasionally the phages were concentrated 10-fold by pelleting during overnight centrifugation at 7,000 × *g*. Transductants were selected on CYE plates containing kanamycin at a concentration of 50 μg/ml. The presence of gene fusion DNA in Km^r transductants was confirmed by colony hybridization (4) by using pMLB1034 as a ^{32}P-labeled probe.

RNA isolation. RNA was isolated from sodium dodecyl sulfate lysates of *M. xanthus* cells by phenol extraction as described previously (2, 4). To isolate *ops* RNA it was necessary to incorporate sonication with glass beads into the procedure to disrupt myxospores (2).

RNase protection assay. RNA synthesis from *tps-lacZ* and *ops-lacZ* fusion genes was assayed by the ability of this RNA to hybridize with ^{32}P-labeled cRNA probes and protect them from RNase digestion (19). The cRNA probes used in this study are shown in Fig. 1 and were synthesized from *Eco*RI-cut pJDK30 (*tps-lacZ*) or *Xba*I-cut pJDK35 (*ops-lacZ*) by using the phage T7 RNA polymerase (Bethesda Research Laboratories). The procedure for the formation of RNA-RNA hybrids, RNase digestion, and analysis of the protected probe RNA on denaturing polyacrylamide gels has been described elsewhere (2, 19). RNA molecules of known size synthesized in vitro served as size standards.

β-galactosidase assays. Cells were harvested from 10-ml samples of vegetative or starvation shaker cultures, and sonicated extracts were prepared as has been described (5). This procedure was modified for the assay of activity from sporulating cultures (late-developmental plate or glycerol-induced cultures) to allow disruption of these sonication-resistant cell forms. The developmental cells from one CF agar plate (diameter, 100 mm) or a 10-ml sample of a glycerol-induced culture were suspended in 1 ml of Z buffer (5, 20) and added to a 2.0-ml microcentrifuge tube, the tube was filled with zirconium beads (diameter, 0.15 mm), and the tubes were agitated vigorously in a Mini-Beadbeater (Biospec Products) in 1-min bursts for a total of 5 min. The glass beads and cell debris were removed by centrifugation before determination of the β-galactosidase activity. The assay has been described elsewhere (20).

RESULTS

Delineation of the *tps* regulatory region. The general approach that was taken to localize the *cis*-acting regulatory regions of the *tps* and *ops* genes was to construct gene fusions in *E. coli* with expression of *lacZ* under the control of various segments of *tps* or *ops* DNA and to analyze the expression of the fusion genes in *M. xanthus* after phage P1-mediated transduction. In all cases the *M. xanthus* DNA included the coding region for the N-terminal 18 amino acids of the *tps* or *ops* protein products with which an in-frame fusion with the *lacZ* product, β-galactosidase, was formed (4, 5). Gene fusions were constructed with various amounts of upstream *tps* or *ops* DNA (Fig. 1) and in some cases with alterations of the wild-type *tps* or *ops* DNA sequence. A key aspect of this analysis was the ability to introduce the fusion genes into *M. xanthus* for expression studies. No plasmids have been characterized which can routinely be used to introduce DNA into *M. xanthus* and study gene expression. Also, while homologous recombination has been used to stably maintain gene fusions in *M. xanthus* (4, 5, 23), this technique was unsuitable, since recombination between the chromosomal *tps* or *ops* genes and fusion gene DNA would reconstruct the intact regulatory region upstream from the fusion gene under analysis (5). Instead, we took advantage of the observation that gene fusion plasmids containing transposon Tn*5* are stably integrated into the *M. xanthus* chromosome after P1 transduction (3). The integrated fusion genes are found at many locations on the bacterial chromosome (regions which do not share homology with the transduced fusion plasmid) and are flanked by Tn*5* or IS*50* sequences, suggesting that they have been transposed to the chromosome under direction of the flanking Tn*5* sequences. Since these fusion plasmids carry small segments of *tps* or *ops* DNA (<420 bp), homologous recombination with the bacterial chromosome has not been observed. In no case has any alteration of transposed gene fusion DNA been observed by Southern blot hybridization analysis.

To investigate the DNA sequences regulating *tps* gene expression, the plasmid pJDK26 was constructed. This plasmid was constructed by first inserting a 201-bp fragment from the *tps* gene (Fig. 1) into the *lacZ* fusion vector pMLB1034 (25) and then isolating a fusion plasmid with a Tn*5* insertion in the ampicillin resistance gene. The *tps* gene DNA in this plasmid extends 95 bp upstream from the transcriptional initiation site (−95). After phage P1-mediated transduction, a number of kanamycin-resistant transductants which contained *tps-lacZ* fusion gene sequences were identified. Expression of the fusion gene was tested initially in eight independent transductants by assaying β-galactosidase activity in lysates of vegetative or 16-h starvation shaker culture (early-developmental) cells. The averaged results from this analysis are presented in Table 1. This fusion gene was clearly expressed at a much higher level during early development than during vegetative growth, indicating that sufficient *tps* gene DNA was present to allow developmental control of gene expression. When the level of expression of this fusion gene was compared with a previously characterized fusion with the intact *tps* upstream region (strain DZF3427) (5), significantly lower developmental activity was observed (90.3 to 301 U). The relatively low level of expression of the pJDK26 fusion gene did not appear to be due to an inherent problem with the system of analysis, since a fusion with almost 1 kbp of upstream *M. xanthus* DNA was expressed at a level comparable to that of the fusion gene in DZF3427 (data not shown). The pJDK26 gene

TABLE 1. β-Galactosidase activity produced from *tps-lacZ* gene fusions

Fusion gene[a]	Sp act[b] (nmol/min per mg) of (cell type):		Dev/veg[c]
	Vegetative	Developmental	
pJDK26	3.3 (1.7)	90 (60)	27
pSK1-5	6.6 (4.2)	181 (95)	27
pSK1-9	3.7 (1.5)	12.3 (4.9)	3.3
pSK1-10	4.6 (2.4)	17.6 (5.7)	3.8
pSK1-11	3.7 (2.7)	12.0 (3.3)	3.2
pSK1-12	14.2 (14.5)	35.3 (28)	2.5
pSK1-13	5.0 (1.7)	13.4 (3.2)	2.7
pSK1-43	4.8 (2.4)	11.5 (3.8)	2.4
DZF3427	1.2	301	250
pSK3	7.0	249	36

[a] Fusion genes are designated according to the *E. coli* plasmids on which they reside (Fig. 1). Strain DZF3427 has a *tps-lacZ* fusion with unaltered *tps* gene upstream DNA (5).

[b] Specific activities were measured in crude lysates of vegetative or 16-h developmental cells. The values reported are averages of the data from eight independent transductants, and the standard deviation is shown in parentheses. Only two pSK3 transductants were tested, and they had nearly equal levels of activity.

[c] Ratio of developmental to vegetative specific activity.

fusion was also expressed at a higher level vegetatively than the DZF3427 fusion (3.3 to 1.2 U). Another characteristic of pJDK26 transductants (and other gene fusion transductants) was the high level of variability of β-galactosidase activity from individual strains, as indicated by the high standard deviation (60 U for developmental activity). Most of the variability is due to the fact that while the majority of the transductants have one copy of the fusion gene, a few have two or more copies and produce correspondingly higher levels of gene fusion activity. It is also possible that the level of expression of particular fusion genes may be influenced by the chromosomal location of the gene (e.g., insertion into a very highly expressed gene). From the analysis of a number of transductants it was clear that patterns of expression of the various gene fusions emerged, as reflected in the average values, and we did not generally try to account for transductants whose expression seemed to deviate from the norm. The time course of expression of the pJDK26 gene fusion during fruiting body formation on developmental agar plates was very similar to that observed during DZF3427 development (data not shown).

RNA from the *tps-lacZ* fusion was also detected by hybridization with a cRNA probe. This probe, shown in Fig. 1, contained both *tps* and *lacZ* sequences and allowed the detection of RNA from both the fusion gene and the resident *tps* gene. When 24-h starvation shaker culture RNA from DZF3427 (intact *tps-lacZ* fusion) was analyzed by the RNase protection assay (19), two prominent sets of protected probe bands were detected (Fig. 2, lane 2). One set of bands, the largest of which is 106 bases, was derived from hybridization with *tps* gene RNA (2), and another, 187 bases, was derived from hybridization with the fusion gene RNA. The size of the protected fusion probe is consistent with the expectation that the 5′ end of the fusion gene RNA maps about 50 bases upstream from the *tps* gene translational initiation codon, as does *tps* RNA. RNA from the *tps* and the *tps-lacZ* genes was not detected in a vegetative DZF3427 RNA preparation (Fig. 2, lane 1). When four pJDK26 transductants were tested for the production of *tps-lacZ* RNA, all were found to have significant levels (Fig. 2, lanes 4, 5, 7, and 8). Although in this experiment a wide range of concentrations of the fusion

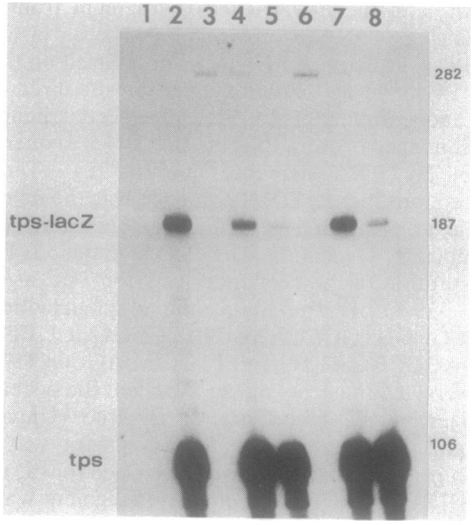

FIG. 2. Production of *tps-lacZ* fusion RNA from several pJDK26 transductants. RNA (10 μg) from vegetative or 24-h developmental cells was analyzed for *tps-lacZ* fusion expression by the RNase protection assay with a cRNA probe to the 5′ end of the fusion RNA (Fig. 1). The test RNA was from *M. xanthus* DZF3427 (which has one copy of a *tps-lacZ* fusion gene with intact upstream DNA sequences) and transductants of pJDK26 (upstream *tps* DNA to −95). Lanes: 1, vegetative DZF3427 RNA; 2, developmental DZF3427 RNA; 3, vegetative JD13 RNA; 4, developmental JD13 RNA; 5, developmental JD14 RNA; 6, vegetative JD15 RNA; 7, developmental JD15 RNA; 8, developmental JD16 RNA. The 187-base band corresponds to the *tps-lacZ* gene transcript, and the set of bands at 106 bases correspond to the *tps* gene transcript. The 282-base band represents full-length protection of the probe and originates upstream from the *M. xanthus* DNA.

gene RNA were observed, two of these strains (JD13 and JD15) were chosen for analysis because they produced relatively high levels of gene fusion enzyme activity and because Southern blotting experiments suggest that these strains contain two and three copies of the fusion gene, respectively (data not shown). The relative amount of fusion gene RNA was consistent with the level of β-galactosidase activity detected in the individual transductants. The *tps-lacZ* RNA was not detected in RNA prepared from vegetative cells (Fig. 2, lanes 3 and 6), but a larger protected probe band (282 bases) was observed. It appears that this RNA is initiated upstream from the *M. xanthus* DNA sequences in pJDK26 (possibly from a Tn*5* promoter), and this may account for the somewhat higher levels of vegetative gene fusion expression that have been observed from the pJDK26 fusion gene and others we have constructed (Table 1). However, it is clear that expression of this fusion gene, containing *tps* DNA upstream to −95, is activated in response to development even though the level of expression is less than that of the *tps* gene itself.

Since the fusion gene in pJDK26 contained sufficient *tps* DNA to be subject to developmental regulation, deletion derivatives of this plasmid were constructed to more clearly define the *tps* regulatory region. All of the deletions started at an *Eco*RI site 7 bp upstream from the *tps* gene DNA and removed various amounts of DNA towards the protein coding sequences. Notably, all the deletions which removed *tps* DNA from position −95 eliminated most, if not all, developmental activation of gene fusion expression (Table 1). The smallest deletion (pSK1-10) removed the segment from −95 to −81, suggesting that this segment plays an

	-95	Regulated expression
tps	--GGGCCTCGGCGCCGACTCCTC--	+
pJDK26	--CTTCAAGAATTCCCACTCCTC--	+
pSK1-5	-----ctctagagCCCACTCCTC--	+
pSK1-9	--ctctagagATTCCCACTCCTC--	-
pSK3	--CGGCCTCGGCGCCGACTCCTC--	+
	-108	

FIG. 3. The DNA sequence immediately upstream from the *M. xanthus* DNA in several gene fusions. The *M. xanthus* DNA extends upstream to −95 in pJDK26, pSK1-5, and pSK1-9 and to −108 in pSK3. An *Eco*RI site is present in pJDK26 (underlined) which was partially deleted in the construction of pSK1-5 and pSK1-9. The underlined bases in pSK1-5 and pSK1-9 indicate the remains of the *Eco*RI site, and the sequence of the *Xba*I linker used in the construction of these plasmids is shown in lower-case letters. The pSK3 fusion is identical to pJDK26 upstream from the *M. xanthus* DNA. The data on regulated expression are from Table 1.

important role in developmental regulation. Surprisingly, the expression of the gene fusion in plasmid pSK1-9 was not developmentally regulated, although it contained the same complement of *tps* DNA as the pJDK26 fusion gene. The alteration in this plasmid occurred upstream from the *M. xanthus* DNA in which the *Eco*RI site was partially removed and an *Xba*I linker was inserted (Fig. 3). Another gene fusion (pSK1-5), with unaltered *tps* DNA and changes at the *Eco*RI site (Fig. 3), was expressed at a higher level than the pJDK26 fusion (Table 1). When 13 bp of additional *tps* DNA was included upstream from the −95 position (pSK3; Fig. 3), two transductants had nearly identical and relatively high levels of regulated fusion gene expression (Table 1).

The relative amount of *tps-lacZ* RNA produced from several of the fusion genes was determined by the RNase protection assay. As shown earlier, less fusion RNA was produced in a representative pJDK26 transductant than in strain DZF3427 (Fig. 4, lanes 1 and 2). A pSK1-5 transductant had more fusion RNA than the pJDK26 strain, and no RNA could be detected from pSK1-9 or pSK1-43 strains (Fig. 4, lanes 3 through 5). All these results were consistent with the relative levels of gene fusion enzyme activity produced in the various strains. Our results clearly indicate

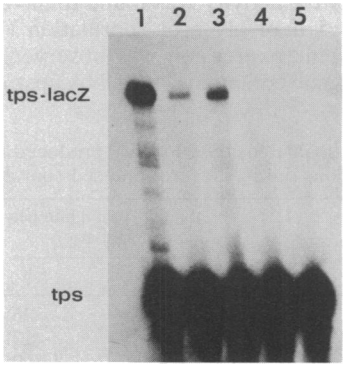

FIG. 4. Developmental *tps-lacZ* fusion RNA expression from deletion derivatives of pJDK26. Developmental RNA (10 μg) from strains DZF3427 (lane 1); JD90, a pJDK26 transductant (lane 2); JD100, a pSK1-5 transductant (lane 3); JD110, a pSK1-9 transductant (lane 4); and JD160, a pSK1-43 transductant (lane 5) was analyzed by the RNase protection assay. The bands corresponding to the *tps-lacZ* and the *tps* gene transcripts are indicated.

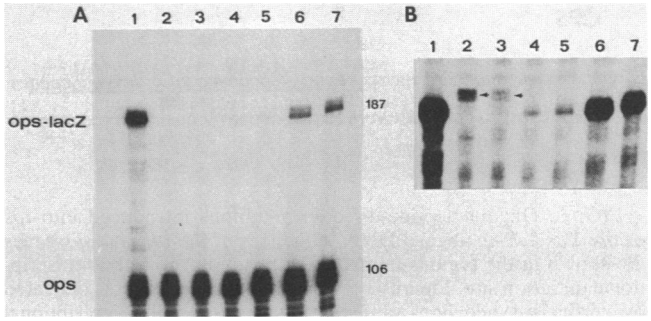

FIG. 5. Developmental *ops-lacZ* fusion RNA expression from fusion genes. RNA was isolated from 64-h developmental cells and analyzed by the RNase protection assay with the *ops-lacZ* cRNA probe (Fig. 1). (A) The RNA (10 μg) which was subjected to this analysis was from strain DZF3361 (lane 1); strains JD40 and JD41 (independent pKS7 transductants) (lanes 2 and 3); strains JD60 and JD61 (independent pKS8 transductants) (lanes 4 and 5); and strains JD70 and JD71 (independent pKS9 transductants) (lanes 6 and 7). The sizes of the bands corresponding to the 5′ ends of the *ops-lacZ* and *ops* RNA are shown at the right in bases. (B) Fourfold longer exposure of the *ops-lacZ* portion of the autoradiogram shown in panel A. A small amount of the specifically initiated *ops-lacZ* RNA was produced in strains JD60 and JD61 (lanes 4 and 5). Arrows indicate bands in lanes 2 and 3 derived from transcripts apparently initiated upstream from the *M. xanthus* DNA in pKS7.

that *cis*-acting DNA sequences upstream from −95 (whether they are *M. xanthus* or vector DNA) influence the level of developmentally regulated *tps* gene expression. However, since several gene fusion constructions which differ in DNA sequence upstream from −95 were found to be developmentally controlled (Fig. 3), our results strongly suggest that *tps* gene DNA downstream from −95 is sufficient to direct specifically the initiation of a substantial amount of RNA synthesis in a developmentally regulated fashion.

Delineation of the *ops* regulatory region. A similar approach was taken to identify the regulatory region of the *ops* gene. In this case *ops-lacZ* fusions were created with different amounts of upstream *M. xanthus* DNA by means of several restriction enzyme cleavage sites in this region. Gene fusions were created with distal endpoints at 9 (pKS7), 131 (pKS8), and 208 (pKS9) bp upstream from the presumptive *ops* transcriptional initiation site (Fig. 1). After transfer of the fusion genes to *M. xanthus*, expression was monitored as described above by assay for β-galactosidase activity and gene fusion RNA. The results of an RNase protection analysis of 64-h (late) developmental RNA from the gene fusion strains are shown in Fig. 5. RNA from strain DZF3661, which contains an *ops-lacZ* fusion with intact upstream *ops* DNA and the entire *ops* gene (4, 5), protected two sets of cRNA probe bands (Fig. 5A, lane 1). The smaller set (about 106 to 109 bases) resulted from hybridization to *ops* gene RNA, and the larger band (187 bases) resulted from fusion RNA hybridization. When late developmental RNA from two separate pKS7 (*M. xanthus* DNA to −9) transductants was analyzed, no *ops-lacZ* RNA with the correct 5′ end was detected (Fig. 5A and B, lanes 2 and 3). However, two slightly larger bands were detected. It appears that transcription initiated upstream from the *M. xanthus* DNA, in vector or Tn*5* sequences, and continued through the gene fusion. There is no indication that this transcription of the pKS7 fusion gene is developmentally regulated (data not shown). A very small amount of *ops-lacZ* RNA was detected in two pKS8 (−131) transductants (Fig. 5B, lanes 4 and 5). The *ops-lacZ* RNA was not detected in 24-h developmental cells,

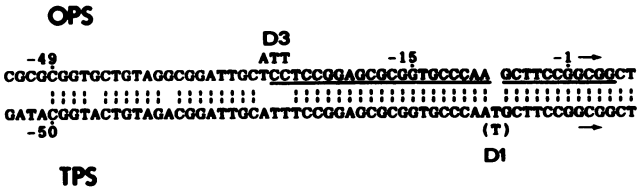

FIG. 6. Oligonucleotide-directed mutations introduced into *tps* or *ops* DNA. The aligned DNA sequences of the *tps* and *ops* genes are shown in the regions immediately upstream from the transcriptional initiation site. Identity between the two sequences is indicated by vertical dashed lines, and the putative sites of transcriptional initiation are shown with arrows. The mutation designated D1 is the deletion of the T (parentheses) at −8 in the *tps* DNA sequence. The D3 mutation is the substitution of ATT for TCC at −27 to −29 in the *ops* DNA sequence.

indicating that the production of the pKS8 fusion RNA was regulated similarly to the *ops* gene (results not shown). Much more developmentally regulated fusion RNA was detected in pKS9 (−208) transductants (Fig. 5A, lanes 6 and 7). Similar results were obtained from assays of β-galactosidase activity from these strains (data not shown).

These results indicate that, while the *ops* DNA sequences downstream from −131 are sufficient for developmentally regulated expression, DNA between −131 and −208 is required for a level of expression which begins to approach that seen for the intact gene. The amount of expression of an *ops-lacZ* fusion gene with more than 600 bp of upstream DNA was similar to that found in DZF3361 (data not shown), suggesting that DNA sequences upstream from −208 play a role in controlling the level of *ops* gene expression.

Site-specific alterations of the *tps* and *ops* gene regulatory regions. The results described above show that the *cis*-acting regulatory regions of both the *tps* and the *ops* genes extend upstream from the DNA segments in which the two genes are related (upstream from about −50). Presumably, this observation accounts, at least in part, for the differential regulation of the genes. However, DNA sequence differences also exist within homologous upstream regions of the two genes, and these sequences may also play a role in differential regulation. Two differences were noted which, because they were located immediately upstream from the putative transcriptional start, appeared important. These sequences were a 1-bp insertion in the *tps* DNA (relative to the *ops* DNA sequence) at position −8, and a 3-bp sequence slightly farther upstream (−27 to −29 in *ops* and −28 to −30 in *tps*) which differed between the two genes (Fig. 6).

To investigate the importance of these DNA sequences in the regulation of the two genes, two alterations were made in the *tps* or the *ops* DNA sequences by oligonucleotide-directed mutagenesis. The changes that were made consisted of the deletion of the T residue at −8 in the *tps* gene (alteration D1) and the conversion of the sequence TCC at −27 to −29 in the *ops* gene to ATT (alteration D3) (Fig. 6). Each of these changes converted the *tps* or the *ops* gene to the sequence of its homolog at the corresponding position. The effects of these changes in the DNA sequence on gene expression were tested by constructing gene fusions containing the alteration and measuring the β-galactosidase activity produced in *M. xanthus*.

The strains with mutations in the *tps* or *ops* regulatory regions showed significant differences in the expression of fusion gene activity compared with fusion genes containing the wild-type regulatory regions. Strains with the D1 alteration carried within a *tps* DNA fragment extending upstream to position −95 produced an average of 33 U of β-galactosidase activity vegetatively and 145 U in 24-h starvation shaker culture (Table 2). When compared with the activity produced by a representative pJDK26 transductant containing the comparable wild-type segment of *tps* DNA, the level of early developmental activity was about the same but vegetative activity was found to be much greater from the mutant fusion gene. Induction of gene fusion expression under the control of the mutant *tps* DNA segment was reduced with a starvation/vegetative activity ratio of 4.4 in contrast to a ratio of 26 for the pJDK26 fusion strain JD90. The D3 alteration in the *ops* DNA segment produced an even more striking result in that very high levels of vegetative activity were observed (93 U). Activity levels increased to an average of 367 U in 24-h early-developmental cells. These values can be compared with 4.1 U and 9.7 U found in vegetative and 24-h starvation culture transductants of the corresponding wild-type *ops-lacZ* fusion (pJDK55). These are conditions under which expression of the *ops* gene is not significantly activated. To test expression of the D3 *ops-lacZ* fusion under conditions in which the *ops* gene is induced, activity was measured during glycerol-induced sporulation. A strain (DZF3631) containing an *ops-lacZ* fusion with intact upstream *M. xanthus* DNA (4, 5) and which expresses a low level of vegetative activity was found to have 17 times more activity after 5 h of glycerol sporulation (Table 3). Less induction of gene expression was observed in strain JD80 with a fusion gene containing *ops* DNA upstream to position

TABLE 2. Effects of site-directed mutations on the expression of gene fusion β-galactosidase activity

Fusion gene[a]	Sp act[b] (nmol/min per mg) of (cell type):		Dev/veg[c]
	Vegetative	Developmental	
pJDK26 (JD90)	6.0	156	26
pJDK26-D1	33 (24)	145 (110)	4.4
pJDK55	4.1	9.7	2.4
pJDK55-D3	93 (27)	367 (99)	3.9

[a] Gene fusions are designated according to the *E. coli* plasmid on which they reside. The pJDK26 transductant was strain JD90. The D1 or D3 plasmids have mutations in the *tps* or *ops* DNA (Fig. 6).

[b] Specific activity was measured in crude extracts of vegetative or 24-h developmental cells. The reported values are averages of the data from transductants for pJDK26-D1 (seven transductants), pJDK55 (two transductants), and pJDK55-D3 (five transductants). Standard deviations are shown in parentheses.

[c] Average developmental specific activity divided by the average vegetative specific activity.

TABLE 3. β-Galactosidase activity produced from *ops-lacZ* fusions during glycerol-induced sporulation

Strain[a]	Sp act[b] (nmol/min per mg) at:		Ind/veg[c]
	0 h	5 h	
DZF3361	1.6	26.8	17
JD80 (pJDK55)	1.4	12.7	9
JD200 (pJDK55-D3)	60.4	1,500	25

[a] Strain DZF3361 has one copy of an *ops-lacZ* fusion with the complete complement of upstream *ops* DNA. Strains JD80 and JD200 are representative transductants of pJDK55 (wild-type *ops* DNA to −311) and pJDK55-D3 (identical to pJDK55 except for the D3 mutation), respectively.

[b] Specific activities were measured in crude lysates of 0-h and 5-h glycerol-induced cells treated so that spores were disrupted.

[c] Ratio of glycerol-induced activity to 0-h (vegetative) activity.

−311. In contrast, a strain (JD200) with the D3 *ops-lacZ* fusion contained 25 times more β-galactosidase activity after 5 h of glycerol sporulation than during vegetative growth (Table 3).

DISCUSSION

The results presented in this study have localized the *cis*-acting regulatory regions of the *tps* and *ops* genes. This has been achieved by constructing *tps* and *ops* fusions with the *E. coli lacZ* gene and analyzing the expression of gene fusion RNA or enzyme activity in *M. xanthus*. In all cases the gene fusions resulted in the production of a fusion protein substituting the N-terminal 18 amino acids of the *tps* or *ops* protein products for the first eight amino acids of β-galactosidase, producing an active enzyme. The expression of the fusion genes is controlled by *tps* or *ops* DNA sequences which extend various distances upstream from the site of fusion. Overall, our results suggest that the upstream regulatory regions of these genes are complex, with elements involved in both positive and negative regulation of developmental transcription.

A fragment of *tps* gene DNA extending 95 bp upstream (−95) from the transcriptional initiation site was found to contain sufficient sequence information to direct the synthesis of developmentally regulated *tps* RNA. This conclusion is based on analysis of the expression of two fusion genes, those found on the plasmids pJDK26 and pSK1-5, which produce developmentally regulated fusion RNA that is initiated at the appropriate position within the *tps* DNA. Although these two fusions have the same complement of *tps* DNA, they differ in plasmid DNA upstream from the *M. xanthus* sequences (Fig. 3). Furthermore, the DNA sequences upstream from the two fusion genes differ greatly from the high G:C *M. xanthus* DNA found upstream from −95 in the intact *tps* gene. These results strongly suggest that the *tps* DNA sequences downstream from −95 are sufficient for regulated expression of the gene. However, it is clear that the DNA sequences upstream from −95 in the fusion gene constructs can influence expression of the downstream fusion. The most obvious example is the pSK1-9 gene fusion, which, although it contains *tps* DNA to position −95, was not expressed developmentally. Also, the pSK1-5 fusion gene was expressed at a higher level than the pJDK26 fusion. Currently, we do not know whether these results are due to differences in one or a small number of important regulatory base pairs which may reside upstream from −95 or to some other reason. Deletions of distal *tps* DNA sequences from −95 towards the protein-coding region resulted uniformly in fusions which were not expressed in a developmentally regulated fashion, suggesting that these deletions (as small as 14 bp) have removed important *cis*-acting regulatory DNA sequences. The region of *tps* gene homology with the *ops* gene ends at position −50, which indicates that nonhomologous DNA sequences are involved in *tps* gene regulation and may be at least part of the basis for differential *tps* and *ops* gene regulation.

Similarly, the *cis*-acting *ops* regulatory DNA sequences extend upstream from −49, the position at which *ops* homology with the *tps* gene terminates. A fusion gene with *ops* DNA upstream to position −131 initiated developmentally regulated transcription of the fusion at the proper site, but only a very low level of expression could be detected. When the *ops* DNA extended to −208, a much higher level of developmentally regulated expression was detected. The DNA segment between −131 and −208 must contain important *cis*-acting regulatory DNA sequences. However, the level of expression observed from the pKS9 fusion gene was still only about one-third that observed in a strain (DZF3361) with an *ops-lacZ* fusion containing unaltered *M. xanthus* upstream DNA. The level of expression observed in DZF3361 was not achieved even when the *ops* DNA reached −311 (unpublished results and Table 3).

With regard to the control of the amount of developmental *tps* and *ops* fusion gene expression, it is clear that DNA segments relatively far upstream are required for maximal expression. These segments have not been carefully defined in this study, but they extend upstream from −108 in the *tps* gene (from analysis of the pKS3 fusion gene) and −311 in the *ops* gene (from analysis of the pJDK55 fusion gene). Fusion genes with much larger segments of upstream DNA, about 1 kbp of *tps* DNA and more than 600 bp of *ops* DNA, were expressed at about the same level as fusions with intact upstream sequences (data not shown). These results may indicate that regulatory sites influencing the levels of gene expression are present in these far-upstream regions, but effects due to the context of the *M. xanthus* DNA in these gene fusion constructions are also possible.

We have also begun to identify specific DNA sequences involved in the regulation of the *tps* and *ops* genes. Initially, our studies have focused on DNA sequences slightly upstream from the transcriptional initiation sites for the two genes which, although they lie within the regions of homology, differ between the genes. The importance of the *tps* sequences at −8 (T) and the *ops* sequence at −27 to −29 (TCC) was investigated by changing each of these sequences to that found at the corresponding position of the opposite gene. Those changes include the deletion of the T at −8 in the *tps* gene (D1) and the substitution of the sequence ATT for TCC at −27 to −29 in the *ops* gene (D3). It is interesting that both of these DNA sequence changes (D1 and D3) substantially increased the level of vegetative expression of fusion genes (Table 2). The largest increase was observed when the D3 alteration was made in the *ops* DNA sequence (>20-fold), but a large increase in vegetative *tps-lacZ* activity was also observed when the D1 alteration was made (>5-fold). It is not clear to what degree the *tps-lacZ* fusion gene with the D1 change is responding to the signals which activate developmental expression. The expression of this fusion gene increased only 4.4-fold during development (Table 2). Since two- to fourfold increases in β-galactosidase specific activity have been observed for noninducible fusion genes (Table 1), it appears that these DNA sequence alterations have affected the ability of the fusion genes to respond to developmental activation signals. In contrast, the expression of the *ops-lacZ* fusion with the D3 alteration was found to still be glycerol inducible despite its high basal level (Table 3). Apparently the *cis*-acting *ops* DNA controlling developmental induction has not been affected by this DNA sequence change.

Clearly the DNA sequences of the *tps* and *ops* genes, which have been altered by the D1 and D3 mutations, are involved in the regulation of these genes. The simplest explanation for the increases in vegetative expression which these two DNA sequence alterations have caused is that the mutant genes may no longer respond to negative regulatory systems that normally limit vegetative expression. It is interesting that a 32-bp inverted repeat sequence is located at the transcriptional start of the *ops* gene (underlined in Fig. 6). This imperfect inverted repeat sequence (18 of 32 bp) is located from −28 to +4 relative to the putative transcriptional start and, by analogy to the operators for classical

repressor proteins, could form a protein binding site. Two of the *ops* base pairs altered by the D3 mutation fall within the upstream border of this inverted repeat sequence and, as a consequence, weaken the repeat. In the corresponding region of the *tps* DNA sequence this inverted repeat sequence is less perfect as a result of the DNA sequence differences between the two genes. But the D1 alteration in this region of the *tps* DNA sequence also increased the level of vegetative *tps-lacZ* expression, suggesting that this segment may also play a role in negative control of *tps* gene expression. A vegetative DNA binding activity which specifically recognizes the *tps* gene regulatory region has been identified, but the binding site for this activity has not been precisely located (G. Brown and J. Downard, manuscript in preparation). DNA binding assays and footprinting experiments should be helpful in identifying and characterizing the *tps* and *ops* gene binding activities in vegetative *M. xanthus* cells and in determining their roles in developmental regulation of gene expression.

The regulatory regions of the *tps* and *ops* genes extend upstream into segments of DNA which are unrelated between the two genes. Currently, there is little information as to the role these upstream regions might play in controlling gene expression. However, the observation that gene fusions lacking important segments of these upstream regulatory DNA sequences were not developmentally inducible suggests that they may be involved in positive regulation. Further studies will be necessary to confirm this notion.

The localization of the *cis*-acting regulatory regions of the *tps* and *ops* genes is an important step towards identifying regulatory proteins and genes involved in the control of gene expression during *M. xanthus* fruiting body formation. Since regulation of these genes is apparently controlled by signals passed between cells, we ultimately hope to understand the specific mechanisms of signal transduction involved. In addition, the high degree of relatedness of the *tps* and *ops* genes provides an interesting opportunity for comparing the distinct regulatory systems responsible for controlling the expression of these genes.

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