Evidence that \textit{asgB} Encodes a DNA-Binding Protein Essential for Growth and Development of \textit{Myxococcus xanthus}

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\textit{Myxococcus xanthus} is a gram-negative soil bacterium that responds to nutrient deprivation by forming spore-filled fruiting bodies. Tens of thousands of cells aggregate to form haystack-shaped structures within which the cells differentiate to form myxospores. Myxospores are metabolically quiescent and are resistant to a number of environmental extremes (for recent reviews, see references 6 and 40).

Cell-cell communication is required to coordinate fruiting-body development, and at least four extracellular signaling events are implicated in this process. Four classes of conditional developmental mutants (\textit{asg}, \textit{bsg}, \textit{csg}, and \textit{dsg}) that fail to develop unless they are in mixture with wild-type cells have been identified; wild-type cells supply the extracellular signal that the mutants fail to produce (10, 18). The correct timing and level of expression of developmentally regulated genes depends on the four sequential cell-cell interactions. As a result, each mutant class is blocked at a different stage of development (17, 18).

The \textit{asg} (A-signaling) mutants are arrested early in development, before the cells have formed aggregates (22). These mutants fail to release A-signal, which is a mixture of amino acids generated by extracellular proteolysis (24, 34). A-signal functions as a cell density signal and is important for \textit{M. xanthus} development because tens of thousands of cells are required to build a fruiting body (23). The presumed function of the fruiting body is to ensure that upon germination, a sufficiently high concentration of cells will be present to maximize cooperative feeding.

Three \textit{asg} loci are known: \textit{asgA}, \textit{asgB}, and \textit{asgC} (21). The \textit{asg} mutants fail to produce extracellular substances other than A-signal, and mutants isolated on the basis of defective protein secretion show a partial \textit{asg} phenotype (20, 31). This finding has led to the hypothesis that the \textit{asg} genes encode parts of the hypothetical export machinery, resulting in a failure to export proteases that generate A-signal, or they encode regulators of genes encoding exported proteins or components of the export machinery (23).

An additional clue to the function of the \textit{asg} genes comes from the results of phenylalanine limitation experiments. Phenylalanine limitation is sufficient to stop growth and induce development of wild-type cells. The \textit{asg} mutants, with the exception of DK412 (\textit{asgA412}), continue vegetative growth when phenylalanine is limiting, as if the mutants fail to sense the starvation condition (25). The phenotypic properties of the \textit{asg} mutants suggest that sensing and/or responding to starvation and the production of extracellular proteins are mediated by the \textit{asg} genes.

Here we present evidence that \textit{asgB} encodes a DNA-binding protein essential for growth and development of \textit{M. xanthus}. \textit{AsgB} contains a putative helix-turn-helix (HTH) near the C terminus that is very similar to the HTH present in region four of major sigma factors. Region 4 is a highly conserved region among the sigma factors (13) and is responsible for interaction with the −35 sequences of promoters (5, 9, 26, 43). \textit{AsgB} lacks similarity to the conserved region of sigma factors that is required for interaction with core RNA polymerase and −10 recognition; therefore, it is unlikely that \textit{AsgB} functions as a sigma factor. Rather, we propose that \textit{AsgB} is a transcription factor that binds to DNA sequences closely related to the −35 hexamer TTGACA. One role of \textit{AsgB} may be to alter gene expression in response to starvation, resulting in production of extracellular A-signal.

\textbf{MATERIALS AND METHODS}

\textbf{Bacteria, plasmids, and phage.} DK1622 is used as wild-type \textit{M. xanthus} (16). DK101 carries the social motility mutation \textit{sglA1} (14) and is used as the wild type where indicated. DK480 contains the \textit{asgB480} and \textit{sglA1} mutations (10). Plasmids were propagated in \textit{Escherichia coli} DH10B. Plasmid pKAM025 is a pUC18 derivative that contains the \textit{M. xanthus} \textit{asgB} gene on a 3.3-kb BamHI-SphI DNA fragment (29). P1 cam clb-100 was used to introduce pLP22 into \textit{M. xanthus} (42).

\textbf{Growth and development.} \textit{M. xanthus} strains were grown at 32°C in CTT broth (1% Casitone [Difco Laboratories], 10 mM Tris hydrochloride [pH 8.0], 1 mM KH₂PO₄ [pH 7.7], 8 mM

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MgSO4) or on CTT agar (CTT plus 1.5% Bacto Agar [Difco Laboratories]) supplemented with kanamycin sulfate (Sigma) to 40 µg/ml when appropriate. CTT soft agar is CTT plus 0.7% Bacto Agar. CTT agar plates were supplemented with adenine hydrochloride (United States Biochemical Corp.) to 100 µg/ml for use in the gene replacement studies. Development of M. xanthus was carried out on TPM agar (same as CTT agar without the Casitone).

DNA manipulations. Standard procedures for restriction endonuclease digestions, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligations, and other cloning-related techniques were used as described by Sambrook et al. (37). The primers 5'-GGTCTAGATCTCTTC CC-3' and 5'-CCCTGGAGCATCAA-3' were used to amplify asgB480 chromosomal DNA from DK480. Chromosomal DNA was prepared as described by Avery and Kaiser (2).

DNA sequence determination and computer analysis. DNA sequence was determined by the dideoxy-chain termination method (38), using Sequenase version 2.0 (United States Biochemical) and custom oligonucleotide primers synthesized by the DNA Technologies Laboratory at Texas A&M University. The DNA sequence was assembled and analyzed by using software from the Genetics Computer Group (1991). Codon usage was analyzed using CodonUse 3.0 (1993). GenBank searches were performed at the National Center for Biotechnology Information, using the BLAST network service.

RNA isolation and hybridization. M. xanthus cells were grown to a density of approximately 5 × 10^8 cells per ml in 200 ml of CTT broth. This provides enough cells to isolate RNA for one time point. The cells were collected by centrifugation at 4,400 × g for 15 min at 4°C. For isolation of RNA from growing cells (t = 0), the cell pellet was resuspended in 4 ml of ice-cold 10 mM sodium acetate (pH 5.0), and the hot phenol method (27) was used (with modifications) as described below. To collect developing cells for RNA isolation, the cell pellet was resuspended in TPM liquid to a density of 5 × 10^9 cells per ml. Ten milliliters of cells was spotted in 1-drop aliquots, using a 10-ml pipet, onto three 15-cm-diameter TPM plates. The plates were incubated at 32°C for the desired length of time. The developing cells were harvested by scraping the cells from the agar surface with a razor blade and placing them in 50-ml centrifuge tubes. The cells were resuspended in 4 ml of ice-cold sodium acetate (pH 5.0). Sodium dodecyl sulfate was added to 1%. The cells were mixed by vortexing and incubated for 10 min at 65°C. A 3.5-ml aliquot of hot phenol (65°C, saturated with 10 mM sodium acetate [pH 5.0]) was added, and the incubation was continued for 5 min with occasional vortexing. After cooling on ice for 5 min, the mixture was centrifuged for 15 min at 4°C at 12,000 × g. The upper aqueous phase was transferred to a clean 50-ml centrifuge tube, and the hot phenol extraction was repeated twice. An equal volume of chloroform was added to the aqueous phase, and the mixture was centrifuged for 5 min at 12,000 × g. The upper aqueous phase was transferred to a clean tube, and the chloroform extraction was repeated. Ten-volume of 3 M sodium acetate (pH 5.5) and 2.2 volumes of 100% ethanol were added. The slurry was stored in 0.5- to 1.0-ml aliquots at -20°C.

RNA hybridization was performed by a method described by Stephens et al. (45), with modifications communicated by H. Kaplan. During capillary transfer of the RNA, 10 × SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was used rather than 20 × SSC. The prehybridization was carried out in 5 × SSC–1% sodium dodecyl sulfate–50% formamide–5% polyethylene glycol 2000. The labeled DNA probe was added following the prehybridization period, along with sheared, denatured salmon sperm DNA (10 µg/ml of hybridization fluid). DNA probes were labeled by using random oligonucleotide primers (37) purchased from Sigma Chemical Co.

Construction of an asgB-lacZ translational gene fusion and assay for β-galactosidase. The asgB-lacZ translational gene fusion was constructed as follows. Plasmid pKAM025 (29) was digested with XhoI, and the 1.8-kb fragment containing 76% of the asgB open reading frame (ORF) and 1.5 kb of upstream DNA was isolated. This fragment was ligated into the SaI site of pBG518 (44), a kanamycin-resistant analog of pUC18, to form plasmid pLP20. The M. xanthus DNA within this plasmid is oriented such that the end within asgB is nearest the BamHI site within the multiple cloning sequences. A 3-kb DNA fragment containing the lacZ gene cassette was cut out of plasmid pMC1871 (39) with SaI. The ends were filled in using the large fragment of DNA polymerase I (Klenow enzyme). Plasmid pLP20 was cut at the single BamHI site, and the ends were filled in with Klenow enzyme. The linearized and linearized pLP20 were ligated, forming plasmid pLP21. The DNA sequence was determined across the junction of asgB and lacZ, and the two genes were found to be fused in frame, as expected (data not shown). The final step was the insertion of the asgB-lacZ fusion gene into plJ549. Plasmid pLJS49 is a pBR322-derived plasmid that contains the Km' gene and IS50L from Tn5, P1-specific incompatibility, and the Ms8 attP for insertion into the M. xanthus chromosome at att (41). Plasmid pLJS49 was cut at its single EcoRI site, the ends were filled in, and the linearized plasmid was cut with HindIII. Plasmid pLP21, which contains the asgB-lacZ fusion gene, was cut with KpnI, the ends were made blunt by using T4 DNA polymerase, and the linearized plasmid was cut with HindIII. The 4.8-kb fragment from pLP21 and the 21-kb fragment from pLJS49 were ligated to form plasmid pLP22.

Plasmid pLP22 was introduced into M. xanthus DK101 by P1 transduction as described by Shimkets et al. (42). Quantitation of β-galactosidase during growth and development was performed as described by Kroos et al. (19).

Construction of plasmids for gene replacement studies. Derivatives of pKAM025 that contain the Km' gene from pK7 (35) were constructed as follows. Four separate aliquots of pKAM025 DNA were digested with either DraIII, NheI, NotI, or BstXI. These enzymes make single cuts in pKAM025 at sites within the M. xanthus DNA. The ends of the linearized DNAs were made blunt by using T4 DNA polymerase or Klenow enzyme. DNA containing the Km' gene was prepared by digesting pK7 with HindIII and XhoI and filling in the ends. The 1.7-kb fragment containing the Km' gene was purified. The Km' gene was ligated to the linearized pKAM025 DNAs. Purified plasmids were introduced into M. xanthus by electroporation.

Electroporation of M. xanthus. Electroporation of M. xanthus for the gene replacement studies (Fig. 5) was carried out by using a procedure communicated by P. Hartzell and P. Youderian. To prepare cells for electroporation, M. xanthus cells (DK101) were grown in 10 ml of CTT broth to a density of approximately 5 × 10^8 cells per ml. Cells (1.5 ml) were collected by centrifugation at room temperature. The cells were washed three times with 1 ml of double-distilled water and resuspended in double-distilled water to a final volume of 40 µl. A Bio-Rad Gene Pulser was used for the electroporation. The settings used were 0.65 kV, 25 µF, and 400 Ω. Approximately 1 µg (1 to 2 µl) of plasmid DNA purified by alkaline lysis followed by CsCl-ethidium bromide gradient centrifugation (37) was added to 40 µl of cells prepared as described above. Electroporation was carried out in a 0.1-cm
RESULTS

Nucleotide sequence of asgB and flanking DNA. The M. xanthus asgB gene had been localized to a 3.3-kb BamHI-SphI DNA fragment by Mayo and Kaiser (29) and was deduced to span an XhoI site within this fragment. We determined the nucleotide sequences of both strands of the BamHI-SphI fragment (Fig. 1). An ORF spans the XhoI site and extends from nucleotide 1586 to 2074. This ORF of 163 codons has a high percentage of G+C nucleotides (88%) in the third position of the codons and exhibits a codon preference typical for M. xanthus (data not shown), indicating that it is likely to be a protein-coding sequence (3). The calculated molecular weight of the predicted AsgB polypeptide is 18,755. A second ORF, also judged to be a protein-coding sequence by the same criteria, begins 106 bp downstream of the asgB stop codon and continues through the end of the nucleotide sequence. We detected two ORFs upstream of asgB; however, it is unclear whether they encode proteins. One begins at nucleotide 1028, ends at nucleotide 1540, and has 82% G+C nucleotides in the third position of the codons. The original upstream ORF begins at nucleotide 255, ends at nucleotide 707, and has 85% G+C nucleotides in the third position of the codons.

Detection of a potential HTH DNA-binding motif within the deduced amino acid sequence of AsgB. The amino acid sequence of AsgB was deduced from the nucleic acid sequence. A potential HTH DNA-binding motif was detected near the C terminus of the amino acid sequence (Fig. 1 and 2), using the weight matrix method for HTH detection developed by Dodd and Egan (4). The estimated likelihood that the potential HTH in AsgB is a bona fide HTH motif is very high (standard deviation score = 6.1).

Sigma factor region 4 is one of the two most highly conserved regions among the sigma factors and is characterized by its location at the C terminus and a conserved HTH. It is believed that the conserved HTH directly contacts the -35 region of promoter sequences (5, 9, 26, 43). The potential HTH within AsgB is most similar to the HTH found in region 4 of the major sigma factors. Figure 2 shows an alignment of AsgB with conserved region 4 of the major sigma factors from Bacillus subtilis, E. coli, and M. xanthus. Of the 22 amino acids that make up the putative HTH of AsgB, 14 are identical to those of the major sigma factors. No other similarities to sigma factors were detected in any of the reading frames.

We compared the amino acid sequence of the putative AsgB HTH with 91 sequences that make up the master set of HTH sequences used by Dodd and Egan (4) to evaluate potential HTH motifs. The non-sigma HTH sequences in the master set that are most similar to the 22-amino-acid HTH motif in AsgB are the Drosophila melanogaster homeobox protein PRD (eight identities) and the E. coli transcription control protein CAP (seven identities). The majority of the HTH sequences are 9 to 18% identical to the putative AsgB HTH (data not shown).

Deduced amino acid sequence of the downstream ORF and comparison to PurH. The ORF downstream of asgB extends 375 codons and presumably continues beyond the end of the sequenced DNA (Fig. 1). The deduced amino acid sequence of the downstream ORF was found to be 51% identical to the first 386 amino acids of PurH from E. coli (1, 8) and 48% identical to the first 377 amino acids of PurH from B. subtilis (7) (data not shown). PurH is a bifunctional enzyme that catalyzes the last two steps in the de novo synthesis of IMP (1, 8). Since E. coli PurH and B. subtilis PurH are 529 and 512 amino acids in length, respectively, it is likely that the M. xanthus PurH ORF continues for approximately 140 to 150 codons.

In E. coli, eight of the nine operons that encode the enzymes for purine nucleotide synthesis, as well as other genes involved in the biosynthesis or salvage of purines and pyrimidines, are coregulated by the purine repressor (PurR) (12). PurR binds to a highly conserved 16-bp sequence (CGCGAACGTTT CgTtG) located near the -35 sequence to negatively regulate genes that contain this motif (1, 36). These sequences have been named PUR boxes (46). We searched our DNA sequence for a PUR box sequence even though we do not know whether a Pur repressor that binds to a similar sequence exists in M. xanthus. A sequence identical to the first 8 bp of the PUR box begins 26 bp upstream of the putative -35 sequence of the M. xanthus purB gene (Fig. 1).

Identification of the asgB480 mutation. Mayo and Kaiser (29) determined the approximate location of the mutation within asgB480. All plasmids capable of correcting the asgB mutant phenotype of DK480 (sglA asgB480) in rescue experiments have in common a 400-bp Smal-Xhol DNA fragment (Fig. 1). The asgB480 mutation was determined by sequencing PCR products obtained by using chromosomal DNA from DK480 and two primers, one upstream of the Smal site and one downstream of the Xhol site (see Materials and Methods). The sequences of both strands were determined. A transition mutation (A to G) at position 1664 was found within the 400 bp of DNA between the Smal and Xhol sites. This mutation causes a threonine-to-serine change in the predicted AsgB protein sequence (Fig. 1).

Analysis of asgB mRNA accumulation during growth and development. M. xanthus cells containing the asgB480 allele have an altered growth phenotype as well as an altered developmental phenotype. Growing asgB480 cells are tan rather than bright yellow, and they are less cohesive than asg+ cells (21). Mayo and Kaiser (29) prepared RNA from growing M. xanthus cells and found two size classes of RNA, approximately 0.5 and 0.65 kb, that hybridize to asgB DNA probes. We prepared RNA from growing cells, as well as cells at 2, 4.5, 7.5, and 17.5 h of development, for analysis of asgB mRNA accumulation. The RNA was probed with M. xanthus DNA that extends from the Smal site 35 bp upstream of the AsgB ORF to the Xhol site within the region encoding the putative HTH (Fig. 1). This probe hybridizes to two size classes of RNA, approximately 0.5 and 0.65 kb, that are present in growing cells as well as in developing cells (Fig. 3). The amount of hybridization is similar for all RNA preparations (although a slight increase is seen with the 4.5-h RNA), indicating that there is little relative change in asgB mRNA levels during development. The sizes of the RNAs suggest that there is
heterogeneity at the 3′ and/or 5′ ends and that asgB transcription terminates immediately downstream of the ORF.

Expression of an asgB-lacZ translational fusion. We constructed an asgB-lacZ fusion gene in E. coli in order to study expression of asgB during growth and development. The gene fusion was introduced into the vector pL21B, which contains the LacZ reporter gene. The plasmid was then transformed into E. coli, and the fusion gene was expressed in the resulting strain. The expression levels were measured using an optical density assay. The results showed that asgB-lacZ expression increases significantly during growth, reaching a maximum at the early stages of development. The induction of asgB-lacZ expression was found to be dependent on the promoter region of the asgB gene.

Gene replacement studies. We constructed plasmid derivatives of pKAM025 that contain a kanamycin resistance marker for use in gene replacement studies. A 1.7-kb fragment containing the Km′ gene from pKAM025 was inserted into pKAM025 at the DraIII, NheI, NotI, and BstXI restriction enzyme sites (Fig. 5). Since the plasmids do not replicate autonomously in M. xanthus, they must integrate into the chromosome by single or double crossover in order to confer kanamycin resistance. A single-crossover event would lead to the formation of a tandem duplication with vector sequences located between the two copies of asgB DNA. A double-crossover event (one crossover on each side of the Km′ gene) would result in the replacement of chromosomal DNA with homologous DNA containing the Km′ gene. These gene replacement strains would lack vector sequences.

The plasmids described above were introduced into wild-type M. xanthus by electroporation, and Km′ transfectants were picked to rich (CTT) plates containing kanamycin and adenine. Adenine was added because it was expected that gene replacements resulting in formation of a purH null would require adenine; however, the purH nulls were found to grow on CTT plates without adenine supplementation. Colony hybridizations were performed with a vector (pUC19) probe to determine whether gene replacements had occurred; the probe would not be expected to hybridize to colonies containing gene replacements. The probe did not hybridize to a control Km′ strain lacking vector sequences (data not shown). Gene replacements were obtained with use of the plasmids that contain the Km′ gene outside of asgB. However, no replacements were obtained with use of the plasmid containing the Km′ gene within asgB (Fig. 5). These results strongly suggest that asgB is essential for growth. It is interesting in this respect that asgB480, which is the only mutant asgB allele that has been isolated (21), contains a mutation that results in a single amino acid substitution within AsgB.

DISCUSSION

We predict that AsgB is a transcription factor that recognizes a DNA sequence closely related to the −35 hexamer (TTGACA). Our reasoning is as follows. First, it is highly likely that AsgB contains a DNA-binding HTH. A large number of proteins involved in transcriptional regulation recognize specific DNA sequences through DNA-binding HTH domains (11). Second, the putative HTH of AsgB is more similar to the HTHs found in region 4.2 of sigma factors, particularly the major sigma factors, than to all other HTHs in the database. Region 4.2 is highly conserved among the major sigma factors and participates in recognition of the −35 hexamer of promoters (26). Of the 22 amino acids that make up the HTH motif of AsgB, 14 are identical to those of the major sigma factors of E. coli, B. subtilis, and M. xanthus (Fig. 2). There are no other non-sigma proteins we know of that have this high degree of similarity to the major sigma factor HTH. Members of the FixJ family of prokaryotic transcription factors have conserved C-terminal domains that are similar to region 4 of sigma factors (15); however, they are not specifically similar to...
the major sigma factors. Third, the putative recognition helix within the AsgB HTH is identical to the recognition helix of σ^70 at seven of nine positions, including arginines 584 and 588, which are thought to make direct contact with the −35 hexamer (9, 43). One of the two nonidentities is a conservative change. Finally, there is substantial physical and genetic evidence that the HTH within region 4.2 of sigma factors makes direct contact with, and is responsible for specific recognition of, the −35 region of promoters (5, 9, 26, 28, 43). The high degree of similarity between the putative HTH of AsgB and the HTH of major sigma factors leads to the hypothesis that AsgB binds to TTGACA or a closely related sequence.

If AsgB binds to −35 regions and makes contacts similar to those made by sigma, it may repress transcription by competing with the major form of RNA polymerase holoenzyme for specific binding to promoters. Two simple models based on this repressor/competitor hypothesis are (i) AsgB represses transcription of an early class of developmental genes during growth and is inactive during early development (model 1) and (ii) AsgB represses transcription of vegetative genes during development (model 2). We favor model 1 because it can account for the essential nature of asgB if we assume that transcription of the early class of developmental genes is lethal.

The lack of apparent regulation of asgB expression, indicated by our mRNA analysis and asgB-lacZ expression studies, implies that asgB is regulated posttranslationally. It is interesting in this respect that the mutation in asgB-480 causes a threonine-to-alanine substitution at position 27, which is within a potential phosphorylation site for serine/threonine kinases that recognize the amino acid sequence RXXS/T (Fig. 1). The M. xanthus pknl gene encodes a serine/threonine kinase required for normal development (30), and there is evidence that at least 26 different genes encoding eukaryotic-like protein kinases exist in M. xanthus (47). Perhaps asgB-480 cells fail to develop because AsgB-480 cannot be phosphorylated in response to starvation. Returning to model 1, we would predict that unphosphorylated AsgB represses the early class of developmental genes during growth; upon starvation, AsgB is phosphorylated and allows expression of the developmental genes.

Clearly, there are alternatives to the repressor/competitor hypothesis. AsgB may be an activator or repressor that binds to nonpromoter sequences that resemble the −35 hexamer. Second, AsgB may be part of a multicomponent sigma factor that can interact with core RNA polymerase. AsgB presumably would provide specificity for the −35 region, while other proteins would allow interaction with RNA polymerase or other promoter sequences.

In summary, our results provide evidence that AsgB is a DNA-binding protein essential for growth as well as A-signal production. Since asgB-480 cells appear to be defective in exporting a number of proteins (25, 33), including those required for A-signal production, it seems likely that AsgB directly or indirectly regulates expression of genes encoding proteins destined for export or genes encoding components of the export machinery. LaRossa et al. (25) found that the asg mutants continue vegetative growth under conditions of phenylalanine limitation, which suggests that wild-type asg genes are required to respond to starvation conditions. We suspect
that the asg mutants fail to generate extracellular A-signal because they are defective in the fundamental processes of sensing and responding to starvation.

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