Characterization of *bcsA* Mutations That Bypass Two Distinct Signaling Requirements for *Myxococcus xanthus* Development

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The BsgA protease is required for starvation-induced development in *Myxococcus xanthus*. Bypass suppressors of a *bcsA* mutant were isolated to identify genes that may encode additional components of BsgA protease-dependent regulation of development. Strain M951 was isolated following Tn5 mutagenesis of a *bcsA* mutant and was capable of forming fruiting bodies and viable spores in the absence of the BsgA protease. The Tn5Ω951 insertion was localized to a gene, *bcsA*, that encodes a protein that has significant amino acid similarity to a group of recently described flavin-containing monooxygenases involved in styrene catabolism. Mutations in *bcsA* bypassed the developmental requirements for both extracellular B and C signaling but did not bypass the requirement for A signaling. Bypass of the B-signaling requirement by the *bcsA* mutation was accompanied by restored expression of a subset of developmentally induced *lacZ* fusions to the BsgA protease-deficient strain. *bcsA* mutant cells developed considerably faster than wild-type cells at low cell density and altered transcriptional levels of a developmentally induced, cell-density-regulated gene (Ω4427), suggesting that the *bcsA* gene product may normally act to inhibit development in a cell-density-regulated fashion. Bypass of the requirements for both B and C signaling by *bcsA* mutations suggests a possible link between these two genetically, biochemically, and temporally distinct signaling requirements.

Coordinating the activities of large groups of cells during the formation of multicellular structures was once held to be a fundamental problem that is commonly encountered by higher eukaryotes but only rarely by prokaryotic organisms. In recent years, there has been a realization that many bacteria exist within interactive communities (i.e., biofilms) in which cell-cell signaling coordinates multicellular activities. A growing number of cases are being recognized in which cell-cell communication within the bacterial community is required for activities such as successful colonization of certain surfaces and the interactions of pathogenic and symbiotic bacteria with their respective host.

Myxococcus xanthus is a gram-negative bacterium that exhibits an elaborate multicellular development and is an excellent organism for studying the ways in which large numbers of cells organize and coordinate their behaviors within a biofilm community. When *M. xanthus* encounters nutrient-limiting conditions, provided the cells are at sufficiently high density on a solid substrate, the developmental program is initiated. In this process, cells aggregate by coordinated and directed movement to form macroscopic fruiting body structures, each containing a few hundred thousand cells (12, 13, 25, 44). Within the fruiting body, a portion of each cell differentiates into dormant, environmentally resistant myxospores. Upon return to nutrient-sufficient conditions, the myxospores germinate to form viable, growing cells.

The *M. xanthus* developmental program proceeds through an orderly progression of differential gene expression that is

strictly dependent upon signaling between cells (14, 29, 33). The essential role of intercellular signaling in development has been deduced from the behavior of a group of conditional developmental mutants which are believed to be defective in various intercellular signaling steps (8, 10, 17). These mutants failed to develop when tested under standard conditions, yet sporulation was rescued when tested in mixtures with wild-type cells, a behavior termed “extracellular complementation.” Mutants of this type were divided into five classes, based on the results of pairwise mixing of individual mutants. Pairs of mutants in which development was restored were assigned to different signaling groups (designated Asg for A signal, Bsg for B signal, etc.) and are thought to identify five discrete signaling steps that are required for development. For two of the signaling groups, Asg and Csg, putative intercellular signals have been identified and partially characterized (9, 30, 34, 35).

The developmental program of each of five classes of signaling mutants is blocked at a characteristic developmental stage based on morphological and molecular criteria (7, 10, 29, 36). The study of the regulation of developmental gene expression has been greatly facilitated by the use of a set of thirty-six developmentally induced genes identified by Kroos et al. as transcriptional (operon) fusions to *lacZ* using the Tn5*lac* transposon (31). Each Tn5*lac* fusion is transcribed at a characteristic temporal stage during the developmental program, and expression of each fusion is blocked in one or more of the classes of signaling mutants (29). In many cases, the expression of the Tn5*lac* is restored by extracellular complementation or by addition of purified signal (11, 14, 27, 29, 33, 35).

Of the B-signaling mutants identified in the Hagen collection (17), a subset has been genetically characterized and found to have mutations in a single gene, designated *bcsA*. (15, 35). The *bcsA* mutants do not form fruiting bodies or spores and so by morphological criteria appear to be blocked very

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TABLE 1. *Myxococcus xanthus* strains

Strain	Description	Source and/or reference
DZF1	Developmentally proficient genetic background used for certain strain constructions; grows dispersed in liquid culture due to an S-motility mutation in <i>pilQ</i>	D. Zusman; 41 and 52
M853	DZF1 <i>bsgA330</i>	19
M951	M853 <i>bcsA::Tn5</i> Ω951	This study
M972	M853 <i>bcsA::Tn5</i> [Tp ^r]Ω951	This study
M1178	DZF1 <i>bcsA::Tn5</i> Ω951	This study
M3009	DZF1 <i>bcsA::Tn5-132</i> Ω951	This study
DK101	Developmentally proficient genetic background used for certain strain constructions; grows dispersed in liquid culture due to an S-motility mutation, <i>sglA1</i>	D. Kaiser
DK731	M101 <i>csgA4731</i>	D. Kaiser; 17
M3011	M101 <i>csgA4731 bcsA::Tn5-132</i> Ω951	This study
M3012	M101 <i>csgA4731 bcsA::Tn5-132</i> Ω951/ <i>bcsA</i> ⁺⁺	This study
M3013	M101 <i>bcsA</i> ⁺ / <i>bcsA</i> ⁺⁺	This study
DK1622	Developmentally proficient genetic background used for certain strain constructions; fully motile	23
LS523	DK1622 <i>csgA205</i>	45
M1187	LS523 <i>bcsA1187</i> ; <i>bcsA1187</i> was introduced by integration of a plasmid containing an internal <i>bcsA</i> restriction fragment	This study
M252	DK1622 <i>bsgA330</i>	15
M1188	M252 <i>bcsA1187</i> ; <i>bcsA1187</i> was introduced as in M1187	This study
M1189	DK1622 <i>bcsA::Tn5</i> Ω951	This study

^a The designation “*bcsA*⁺⁺” denotes the presence of a second copy of the *bcsA* gene cloned in pREG1962 (19) and integrated into the bacterial chromosome at the bacteriophage Mx8 *attB* site.

early in the developmental program. Mutations in *bsgA* either reduced or abolished the expression of each of the developmentally induced *lacZ* fusions that was tested, suggesting that *bsgA* plays a central role in initiating the developmental program (14, 29). The *bsgA* gene encodes an ATP-dependent protease that shares 45% amino acid identity with the *Escherichia coli* Lon protease (16). The BsgA protease substrate(s) critical for regulation of developmental gene expression in *M. xanthus* has not yet been identified.

For the studies presented here, we took a genetic approach to identify the regulatory components of the BsgA protease-dependent regulatory pathway by isolating mutations that bypass the developmental requirement for the protease. In this report, we describe the characterization of one such mutation, Tn5Ω951, which is localized to a gene designated *bcsA* and is predicted to encode a protein that shares a high degree of amino acid similarity with a recently described group of flavin-containing monooxygenases. Restoration of development in the *bsgA* mutant by a *bcsA* mutation was accompanied by restoration of the BsgA protease-deficient strain to near-normal levels of expression of a subset of developmentally induced genes, although expression of many other genes was unaffected. Mutations in *bcsA* not only alleviated the requirement for B signaling and the BsgA protease but also bypassed the requirement for C signaling and restored both fruiting body and spore formation to the *bsgA* and *csgA* mutants. However, the developmental requirement for A signaling was unaffected. These observations suggest the novel and exciting possibility that the B- and C-signaling pathways, although genetically and temporally quite distinct, may be functionally related and share certain common regulatory components.

MATERIALS AND METHODS

Bacteria, bacteriophages, and transposons. The strains of *M. xanthus* used in this study are as listed (Table 1). The *lacZ* fusions used in this study are transcriptional (operon) fusions formed by insertion of the Tn5*lac* transposon (28).

Independent Tn5*lac* insertions are designated by an omega number (e.g., Tn5*lac*Ω4521 [or simply Ω4521]). For strain constructions, individual Tn5*lac* insertions were transferred by generalized transduction and selected for kanamycin resistance.

The *bsgA330*[Tc^r] allele contains a Tn10-derived *Bgl*III fragment of the tetracycline resistance gene that was cloned into the unique *Bgl*III site of the *M. xanthus bsgA* gene (19). Strain M951 was created by Tn5 mutagenesis of the *bsgA330* mutant strain M853, using bacteriophage P1::Tn5 (32). Strain M972 is a derivative of M951 in which the kanamycin resistance of Tn5Ω951 has been replaced with trimethoprim resistance. The Tn5 insertion in M972 remains in precisely the same location as Tn5Ω951, as determined by Southern blot analysis, and confers the same phenotype as the original Tn5Ω951 mutation. Replacement of kanamycin resistance by trimethoprim resistance was performed using P1::Tn5[Tp^r] (obtained from L. Shimkets) as previously described (2). Similarly, strain M3009 is a derivative of M951 in which the kanamycin resistance of Tn5Ω951 has been replaced with tetracycline resistance, using P1::Tn5-132 as previously described (2).

The myxophage Mx4 *hft hrm ts18ts27* (6) was used for strain constructions by using generalized transduction. The coliphage P1c1r100Cm (15, 32) or electroporation (26) was used to transfer recombinant plasmids into *M. xanthus*.

Medium, antibiotics, and buffers. *M. xanthus* cells were grown vegetatively in CTT medium (4) and supplemented with 40 μg of kanamycin sulfate/ml or 12.5 μg of oxytetracycline/ml, as appropriate. Concentrated cells were routinely washed and resuspended in TPM buffer (10 mM Tris, 8 mM MgSO₄, 1 mM K₂HPO₄, adjusted to a final pH of 7.6) for plating on developmental medium. Developmental phenotypes were routinely determined on clone fruiting (CF) agar (4) or TPMP agar (TPM supplemented with 1% pyruvate and 1.8% agar).

Recombinant genetic methods. Chromosomal DNA purification, transformations, and enzymatic methods, including restriction endonuclease digestion, ligation, and DNA modifications, were carried out according to the procedures of Maniatis et al. (37) or the manufacturers' instructions. Plasmids were purified by alkaline lysis and CsCl-ethidium bromide gradient centrifugation. Circular DNAs were electroporated into *M. xanthus* cells according to the procedures of Kashafi and Hartzell (26). DNA sequencing was performed by the 2',3'-dideoxy chain termination method by using Sequenase version 2.0 DNA sequencing reagents (USB) and dGTP analog 7-deaza-dGTP. GenBank database searches were performed using gapped-BLAST software (1) or gapped-BLAST with BEAUTY postprocessing (47, 54). Motifs were identified by comparison to the PROSITE (5, 22) and BLOCKS (20, 21) databases. Multiple alignments were prepared using ClustalW 1.8 (50), and the graphical output was created using BOXSHADE 3.21.

Cloning of the Ω951 locus by inverse PCR. Inverse PCR was used to amplify a DNA fragment containing the Tn5-chromosome junction from strain M951,

using a protocol suggested to us by L. Shimkets. Chromosomal DNA from strain M951 was digested with *NarI*, adjusted to 5 $\mu\text{g}/\text{ml}$, and ligated to circularize the individual restriction fragments. *NarI* cleaves 324 bases from the outer ends of Tn5; as a result, the circularized, junction-containing fragment consists of the 324 bases from the end of Tn5 plus the adjacent *M. xanthus* DNA extending to the next *NarI* site. PCR primers 5' GGTTCGGTTTCAGGACGCTAC (complementary to Tn5 nucleotides 18 to 37) and 5' GGTGATCCTCGCCGTACTGC (Tn5 nucleotides 158 to 177) anneal to Tn5 sequences within the circularized fragment and are oriented toward the Tn5-chromosome junction and the Tn5 *NarI* site, respectively. Although two PCR products, one from the junction of each end of Tn5, are possible, only one product, approximately 300 bp in length, was observed from this reaction. The product was purified, cloned, and sequenced to confirm that it contained the Tn5-chromosome junction. The labeled PCR product was then used as a probe in colony hybridizations to identify individual cosmids containing the Ω 951 locus from a wild-type cosmid library. Restriction fragments from the hybridizing cosmid were cloned and sequenced to generate the complete sequence of *bcsA* and the adjacent downstream gene.

Construction of *csgA bcsA* double-mutant strains. Initial attempts to transduce the Tn5 Ω 951 insertion into the *csgA205* mutant strain (LS523) were unsuccessful. Therefore, an alternative approach was used to create a *csgA205 bcsA* double mutant. An internal *NarI/NcoI* fragment of the *bcsA* gene was cloned into pCR 2.1 (Stratagene) to give pREG-*bcsA*-int. Integration of this plasmid into the *M. xanthus* chromosome is predicted to create a merodiploid structure containing two truncated copies of *bcsA* but no remaining intact copy of *bcsA*. This plasmid was electroporated into LS523 and a *bgsA330* mutant control strain, M252, to form M1187 and M1188, respectively, and the resulting *bcsA* mutant allele was designated *bcsA1187*. The *bcsA1187* mutant genotype of both strains was confirmed by Southern blot analysis.

The *bcsA*::Tn5-132 Ω 951 mutation from strain M3009 was introduced into the *csgA731* mutant strain M731 by generalized transduction and selection for resistance to oxytetracycline to yield the *csgA731 bcsA*::Tn5-132 Ω 951 double-mutant strain M3011.

Complementation of *bcsA* in a *bgsA bcsA* mutant. The *XhoI* and *SstI* fragments from the *bcsA* locus (see Fig. 2A) were cloned into pREG1962 (19), and the resulting plasmids were electroporated into various *bcsA* mutant strains for complementation analysis. pREG1962 does not replicate in *M. xanthus* but rather integrates very efficiently into the bacterial chromosome at the bacteriophage Mx8 *attB* site by site-specific recombination (45, 48), forming a relatively stable merodiploid.

Measurement of β -galactosidase in vegetative cells. β -Galactosidase-specific activity was determined by the rate of ONPG (*o*-nitrophenyl- β -D-galactopyranoside) cleavage in cell-free sonicates of cells harvested at various times during the growth and stationary phases as described previously (31, 40). The specific activity of β -galactosidase is reported as nanomoles of ONP produced per milligram of protein per minute. The concentration of protein in the extracts was determined by the Coomassie blue dye binding assay (Bio-Rad).

Developmental β -galactosidase assay. A single, isolated colony was grown in CTT broth to 5×10^8 cells per ml, washed, and resuspended in TPMP at 5×10^9 cells per ml. Cells were plated on TPMP plates as 10- μl -diameter spots, with 10 spots per plate. Plates were incubated for various lengths of time at 31°C and harvested by gentle scraping of the cells from the agar surface. The specific activity of β -galactosidase in sonic extracts of harvested cells was determined as described above.

Developmental assay. The developmental phenotypes of individual *M. xanthus* strains used in this study were determined by plating cells on CF or TPMP agar. Plates were observed daily for the appearance of fruiting bodies. After 5 to 8 days, plates were also examined microscopically for refractile spores. For quantitation of spore production, cells were scraped from the agar surface, sonicated in TPM buffer to lyse vegetative cells, and enumerated microscopically in a Petroff-Hauser counting chamber. The number of viable spores was determined by plating dilutions of the sonicated spores on nutrient-rich medium (CTT) and then counting the number of colonies that formed after 5 to 8 days.

RESULTS

Isolation of a bypass suppressor of the *bgsA* mutant. To search for additional components involved in the BsgA protease-dependent regulation of development, a scheme was developed to isolate bypass suppressor mutations which allow development of a *bgsA* null mutant. The *bgsA330* mutant M853 was mutagenized with Tn5, followed by screening of individual

FIG. 1. Developmental phenotype of the *bgsA* suppressor strain M951. Strains used in each column from left to right: DZF1 (wild type [wt]), M853 (*bgsA*), and M951 (*bgsA Tn5Ω951*). Cells were plated on CF agar to induce development and photographed at the times indicated. Top row, cells plated as 10- μl spots of a high-density cell suspension (5×10^7 cells per 10- μl spot); middle and bottom rows, cells plated as 10- μl spots of a low-density cell suspension (2.5×10^6 cells per 10- μl spot).

Tn5-containing (Km^r) colonies for their ability to develop when plated on CF agar. Strain M951 was one of several mutants identified that bypassed the developmental requirement for the BsgA protease and was chosen for further studies presented here.

A genetic backcross was performed to ensure that the developmental phenotype of strain M951 was due to the Tn5 insertion itself and not to a spurious mutation that may have occurred during the selection process. The Tn5 insertion from the M951 donor strain (designated Tn5 Ω 951) was transduced into the parental *bgsA330* mutant strain M853. Three transductants were analyzed and found to have a developmental phenotype indistinguishable from that of strain M951. Southern blot analysis of each of the transductants confirmed the presence of the Tn5 Ω 951 insertion at its original position and the presence of the *bgsA330* mutation (data not shown). These results indicated that the phenotype of strain M951 could be attributed to the effects of the Tn5 insertion and that the gene(s) affected by the Tn5 insertion may identify components of the BsgA protease-controlled pathway for development.

Figure 1 (top row, right panel) illustrates the fruiting bodies containing refractile, sonication-resistant spores formed by the suppressor strain M951 when cells were plated on CF agar at high cell density (5×10^7 cells per 10- μl spot). In contrast, the *bgsA* mutant neither aggregated nor formed detectable numbers of spores (Fig. 1, top row, center panel). Under these conditions, the fruiting bodies produced by M951 were somewhat smaller and more numerous than those formed by wild-type cells (Fig. 1, top row, left panel) and the kinetics of development were somewhat delayed (data not shown). Nevertheless, the suppressor strain M951 clearly regained the capacity to form fruiting bodies and spores and so bypassed the developmental requirement for the BsgA protease.

Although the development of strain M951 was delayed relative to that of the wild-type parental strain when plated at high cell density, strain M951 cells developed significantly faster than wild-type cells when plated on starvation agar at low cell density (2.5×10^6 cells per 10- μl spot). After 3 days of development at low cell density (Fig. 1, center row), strain

TABLE 2. Suppression of the sporulation defect in Bsg and Csg signaling mutants by mutations in *bcsA*

Strain	Strain description	% of wild-type numbers of viable spores ^a
DZF1	Developmentally proficient	100
M853	DZF1 <i>bsgA</i> 4330	<0.01
M951	DZF1 <i>bsgA bcsA</i> ::Tn5 Ω 951	9
M1178	DZF1 <i>bcsA</i> ::Tn5 Ω 951	93
M3010	DZF1 <i>bsgA bcsA</i> ::Tn5 Ω 951/ <i>bcsA</i> ⁺	0.23
DK1622	Wild-type	100
LS523	DK1622 <i>csgA205</i>	0.16
M1187	DK1622 <i>csgA205 bcsA</i> 1187	14
DK101	Developmentally proficient	100
DK731	DK101 <i>csgA</i> 731	0.09
M3011	DK101 <i>csgA</i> 731 <i>bcsA</i> ::Tn5-132 Ω 951	12
M3012	DK101 <i>csgA</i> 731 <i>bcsA</i> ::Tn5-132 Ω 951/ <i>bcsA</i> ⁺	0.14
M3013	DK101 <i>bcsA</i> ⁺ / <i>bcsA</i> ⁺	96

^a Numbers of viable spores were determined as described in Materials and Methods. Values are expressed as percentages of the numbers of viable spores produced by the respective developmentally proficient parental strains. Each value is the average of at least two separate experiments using independent bacterial isolates.

M951 had formed dark, spore-filled fruiting bodies while the wild-type strain had produced only translucent aggregates that were devoid of refractile spores. The wild-type strain typically began to produce dark, spore-filled fruiting bodies by day 5, 2 days later than strain M951. By 6 days of development at low cell density (Fig. 1, bottom row), both the wild-type and M951 strains had produced dark fruiting bodies containing refractile sonication-resistant spores, whereas the *bsgA* mutant failed to aggregate or form fruiting bodies.

The viability of spores produced by strain M951 was determined. Strain M951 and its wild-type parent DZF1 were plated on CF agar at high cell density (5×10^7 cells per 10- μ l spot) and allowed to develop for 7 days. Spots were then harvested and sonicated to disperse the spores and lyse vegetative cells. The number of spores was enumerated microscopically and by plating in CTT soft agar, which allowed viable spores to germinate and form colonies. For strains M951 and DZF1, the relative numbers of spores determined by both direct count and plating were comparable, indicating that the spores produced by M951 were fully viable. Under these culture conditions, strain M951 produced nearly a thousand times more viable spores than the parental *bsgA* mutant, M853, and approximately 10% of the number of viable spores obtained from wild-type cells (Table 2). Therefore, although the M951 *bcsA bsgA* double mutant has a somewhat reduced sporulation efficiency relative to that of the wild-type strain, its ability to produce numbers of viable spores three orders of magnitude greater than that produced by the *bsgA* mutant itself demonstrates the ability of strain M951 to successfully bypass the developmental requirement for the BsgA protease.

A *bcsA* mutation bypasses the developmental requirement for C but not A signaling. Given the observation that mutations in *bcsA* bypass the requirement for B signaling and the BsgA protease for development, the specificity of this phenotype was tested by determining whether a *bcsA* mutation can bypass other developmental signaling requirements. To test the requirement for C signaling, a *csgA bcsA* double mutant was constructed by introducing a *bcsA* mutation into the strain

LS523 (*csgA205*). The *bcsA* gene was disrupted by integrating a plasmid containing a cloned internal fragment of the *bcsA* coding sequence into the *bcsA* locus by homologous recombination, resulting in strain M1187. To verify the phenotype conferred by disruption of the *bcsA* gene in this manner, the analogous mutation was created in a *bsgA* mutant and was found to bypass the developmental defect of the *bsgA* mutation, based on formation of fruiting bodies and quantitation of viable spores (data not shown). The developmental morphology and yield of viable spores were determined for the *csgA bcsA* double mutant and its *csgA* parent. As previously described, the *csgA* mutant formed only rudimentary transparent aggregates and less than 0.2% of the number of viable spores formed by the wild type (Table 2). In contrast, the *csgA bcsA* double mutant formed mature, fruiting bodies that contained at least 100-fold greater numbers of viable spores than the *csgA* parent (Table 2). The number of viable spores produced in the *csgA bcsA* double mutant was approximately 10% of the number of spores produced by wild-type cells, nearly the same number of viable spores produced by the *bsgA bcsA* double mutant.

Mutations in *bcsA* do not bypass all of the required developmental signaling steps. After transduction of the *bcsA*::Tn5 Ω 951 insertion mutation into the A-signal-deficient *asgB480* mutant DK480, the phenotype of the resulting double mutant was indistinguishable from that of the *asgB480* parent, based on fruiting body morphology and viable spore production (data not shown). These observations indicate that *bcsA* mutations do not bypass the developmental requirement for A signaling.

Cloning and sequencing of the Ω 951 locus. Inverse PCR was used as described in Materials and Methods to amplify a DNA fragment containing the Tn5 Ω 951-chromosome junction and 93 bp of chromosomal DNA immediately adjacent to the Tn5 insertion. The amplified fragment was used as a hybridization probe to identify a cosmid that contained the Tn5 Ω 951 insertion site and from which a hybridizing 1.5-kb *Sst*I fragment and an overlapping 2.0-kb *Bgl*II fragment were cloned. The two fragments were sequenced, yielding approximately 3 kb of contiguous DNA sequence in the vicinity of the Tn5 Ω 951 insertion (Fig. 2). The precise position of the Tn5 insertion in strain M951 was determined by sequencing the Tn5 Ω 951 inverse PCR product. From these data, we determined that the Tn5 Ω 951 insertion was located within a 1,320-bp open reading frame (ORF) that features the codon bias expected for an authentic coding sequence of an organism, such as *M. xanthus*, with a high G+C content. The ORF contains the sequence GGAGGAA, located immediately upstream of the predicted ATG initiator codon, that is predicted to serve as a strong ribosomal binding site. Based on results that are discussed below, this gene has been designated *bcsA*, for the ability of mutations in the gene to act as a *bsgA* and *csgA* suppressor.

The predicted *bcsA* gene product (BcsA) contains significant homology to a recently described group of monooxygenases (designated StyA) that are involved in the enzymatic degradation of styrene by certain isolates of *Pseudomonas* spp. (e.g., accession numbers CAA04000.1 and CAB06823.1) and are included in the general category of flavin-containing monooxygenases such as *p*-hydroxybenzoate hydroxylase (Fig. 3). When averaged over the entire coding region, the BcsA pro-

FIG. 2. Map of the region surrounding the Tn5Ω951 insertion and complementation of the *bcsA*::Tn5Ω951 mutant phenotype. (A) Arrows indicate the location and direction of transcription of the *bcsA* and *panE* genes, as predicted from the nucleotide sequence. The location of the Tn5Ω951 insertion located within the amino-terminal portion of a *bcsA* is indicated (arrowhead). Restriction enzyme sites are shown as follows: S, *Sst*I; X, *Xho*I; B, *Bgl*II. (B) Complementation of the suppressor phenotype of the *bcsA*::Tn5Ω951 mutant. Cells were plated on CF medium to induce development and photographed after 6 days at 30°C. (Panel a) M853 (parental *bsgA* mutant). (Panel b) M972 (*bsgA* Tn5Ω951 [Tp^r]). (Panel c) M972 (containing the *Sst*I fragment cloned into pREG1962 and integrated at the Mx8 *attB* site). (Panel d) M972 (containing the *Xho*I fragment cloned into pREG1962 and integrated at the Mx8 *attB* site).

tein has 37% amino acid sequence identity and 52% similarity to the StyA monooxygenases from *Pseudomonas* spp. Although the most striking similarity between StyA and BcsA is within the predicted flavin adenine dinucleotide (FAD) binding re-

gion, the predicted BcsA protein also contains significant amino acid similarity (35% identity, 50% similarity) to the *Pseudomonas* spp. StyA throughout the remainder of the protein.

In general, the level of overall amino acid sequence similarity among monooxygenases is rather low (42, 49). One domain that is particularly well conserved is a βαβ motif that is involved in the binding of the FAD cofactor. A predicted FAD binding domain is located near the amino terminus of BcsA and contains each of the 11 residues which are believed to be critical for the formation of this motif (53). Therefore, it is predicted that BcsA also binds FAD as a cofactor.

The *Pseudomonas* spp. StyA monooxygenases are required for the initial step in the catabolism of styrene (3, 43). The *styA* gene is the first gene in the four-gene *styABCD* operon. These four genes are required for the conversion of styrene into phenylacetic acid, which can then be broken down further by host enzymes. Given the striking similarity between the StyA monooxygenase and the predicted BcsA product, the possibility that homologues of the other genes of the *Pseudomonas styABCD* operon are also present in *M. xanthus* was considered. The sequence of the DNA flanking the *bcsA* gene was determined; however, homologues of the *styB*, *styC*, and *styD* genes were not observed. Instead, a coding region exhibiting *M. xanthus* codon bias and having significant amino acid sequence similarity to *E. coli* α-ketopantoate reductase (*panE*) was identified downstream of *bcsA* (Fig. 2A). The predicted ATG translational start codon of the α-ketopantoate reductase homologue is located 155 bp downstream of the *bcsA* stop codon. It is presently unknown whether the putative α-ketopantoate reductase gene is cotranscribed with the *bcsA* gene or whether it influences *M. xanthus* development. The finding of the *bcsA* homolog of *styA* in the absence of homologs for *styBCD* is not unique. An isolated *styA* homolog is also found in the *Streptomyces coelicolor* genome sequence (accession number CAB45631). No sequences similar to the *Pseudo-*

FAD⁺ binding domain

BcsA	1	-MERIAIVGAGTAGLHLGLKLLSHGVPTIYTEQEPFRLRVSRLLNTVAHHAFTMRERILGVHDHWSGPNADMFIYIGIHV
StyA	1	MKKRIIGIVGAGTAGLHLGLFLRQHDVDVTVTDRKPDDEYSGLRLLNTVAHHAFTVQREVALDVNEWPSEEFGEYGHYYV
PHH	1	MKTQVAIVGAGTAGLHLGLLGLLHKGATDNTVTLERQTPDVLGRIRAGVLEQGMVDLLREAGVDRRMARDGLVHGEVEIAFA
BcsA	80	NGGPHFFSLRGRIDSPTIFVDYROYQPRLEAEFVARGGKLEVLFPVLDLVLERIAOCHALMVVATGRNGLTRLFPRVPALS
StyA	81	GGPQPMRFYGDIAKAPSRAYDYRLYQPMMLRALEARGCKFCYDAVSTDDLEGISEQYDLLLVVCTGKYALGKMEKQSENS
PHH	81	GQRRRIDLRLSGGKTVTVYGQTEVTRDLMEAREACGATTVYQAAEVRLHDLQGERFYVTFFERDGERLRRLDCDYIAGCDG
BcsA	160	PHTOPPRMLFPAALLKGVRMQEPFIGNNANLIPGCGEIFESQVVTADCRVPSVLHFAALPGESELSRLSTORYDEDPRAFEAML
StyA	160	EFKPPORALCVGIFKGIKESPTRALTMSISPGHGELIPIPTLSFNGMSTALVLENHIGSDLEIHAHTKYEDDPRAFDLDM
PHH	161	PHGISRQSIPIERLKVFERVYPFG-----WLGLLADTPPVSHELIYANHPRGFALCSQRSATRSRYVQVE--LTEKV
BcsA	240	MNELRRFAPTTYERVDSRSEFGVR-GPMFYLGQSFTPTVRQGWAPLSNGRFALAVGDTHTVNDPVAQGQANAGSASAFALA
StyA	240	LKKLRKHIFPSVAERIDPAEDDLANSLEILGGVVPVRDGHATLNNKGTIIIGEDVQATVDVPVLQGANMASHAAWILG
PHH	232	EDWSDERFVTELKARLPAAEVAEKLVTGFSLEKSLIAPLRSFVVEPMQHGRLFLAG-DAAHIVPPTGAKGLNLAAADSVSTLY
BcsA	319	EHIVTALADNR-PFDEVFCREAEAGSAAATAPATHWTNTLLQPPPHVIDLLAAGSKDARVATAIATAFVTFELILSACA
StyA	320	EEILAHSVYDL-RFIEHLERRRQDRVLCATR-WTNEFLSAFTELPPEFLAFLQVLSLSREMAFEFTDNFNYPERQWDRFS
PHH	311	RLLLKAYRECRGELLERYSAICLRRIWKAER-FSWWMTSVLHRFPDITDAFSQRIQQTTELEYLGESEAGLATIAENYVGLP
BcsA	398	SPENSAAFIARHRFPMVREAAPPSPPLAWHPPEEPKVTVSLTR
StyA	398	SPERIGQWCSQYAPTIAA-----
PHH	390	YEIE

FIG. 3. Amino acid sequence alignment of the predicted *bcsA* product. Amino acid sequence alignment of the predicted *bcsA* product (top lines [BcsA]) with the StyA styrene monooxygenase from *Pseudomonas* spp. (middle lines [StyA]) and *p*-hydroxybenzoate hydroxylase from *Pseudomonas* spp. (lower lines [PHH]). Shaded residues indicate amino acid identity (dark shading) or amino acid similarity (light shading) between at least two of the three aligned sequences. The putative conserved FAD⁺ binding domain is indicated.

TABLE 3. Expression of Tn5-*lacZ* fusions in the *bsgA bcsA* double mutant during development

Fusion	Expression time (h) ^a	B- or C-signaling requirement ^b	Restored expression in <i>bsgA bcsA</i> double mutant ^c
Ω4521	2	B	—
Ω4455	2	B	—
Ω4494	2	B	—
Ω4273	5	B	—
Ω4411	6	B	+
Ω4427	7	B, C	+
Ω4414	12	B, C	+
Ω4435	25	B, C	—
Ω4401	25	B, C	—

^a Expression time is as defined by Kroos et al. (31) as the time after plating on starvation medium that developmental expression of the respective fusion occurs.

^b Indicates requirement for B or C signal for full expression of the respective fusion (29).

^c The peak level of β-galactosidase expression in the *bsgA bcsA* double mutant was comparable to that of the *bsgA* mutant alone. “+” indicates at least a fourfold higher β-galactosidase specific activity in the double mutant; “—” indicates less than a fourfold difference in specific activity levels.

monas styrene monooxygenase small subunit (*styB*) were detected adjacent to the *Streptomyces styA* homolog. The function of the *Streptomyces coelicolor* gene has not been reported.

Complementation of the bypass suppression phenotype of the *bcsA*::Tn5Ω951 mutation by the cloned *bcsA*⁺ gene. To demonstrate that the phenotype attributed to the Tn5Ω951 mutation is due solely to inactivation of the *bcsA* gene and not to polar effects on the expression of any downstream genes, a complementation test was performed using the cloned wild-type *bcsA* gene. The *XhoI* fragment shown in Fig. 2A contains the entire *bcsA* ORF plus an additional 367 bp of sequence upstream from the predicted ATG translational start codon. This fragment was cloned into pREG1962 (19), and the resulting plasmid was electroporated into strain M972 (*bsgA bcsA*::Tn5Ω951[Tmp^r]; Spo⁺). Chromosomal integration of the plasmid at Mx8 *attB*, the bacteriophage Mx8 attachment site, was confirmed by Southern blot analysis. Although strain M972 is BsgA protease deficient, it contains the Tn5Ω951 mutation and thus yields a strain that is phenotypically Fru⁺ (Fig. 2B, panel b) and Spo⁺ (Table 2). However, introduction of the *bcsA*-containing *XhoI* fragment into this strain resulted in a Spo[−] phenotype, which is identical to that of the *bsgA* mutant in that it was unable to form either fruiting bodies (Fig. 2B, panels a and d) or spores (less than 1% of the number of wild-type spores; see the results for M3010 in Table 2). This indicates that the *XhoI* fragment alone is able to complement the *bcsA* mutant phenotype. The phenotype of M972, containing either the cloned *SstI* fragment, encoding a 49-amino acid carboxy-terminal truncated form of *bcsA* (Fig. 2B, panel c), or the vector alone, was unchanged.

By using a similar strain construction, the *bcsA* gene cloned into pREG1962 and integrated into the bacterial chromosome at the Mx8 *attB* site also complemented the phenotype attributed to the *bcsA*::Tn5Ω951 mutation in a *csgA* mutant. As shown in Table 2, introduction of the cloned wild-type *bcsA* gene into the *csgA731 bcsA*::Tn5Ω951 double mutant, M3012, resulted in a sporulation defect comparable to that of the *csgA731* parent itself. However, introduction of the cloned *bcsA* gene into the respective developmentally proficient par-

ent, DK101, had no significant effect (see the results for M3013 in Table 2).

The data presented in this section indicate that suppression of both *bsgA* and *csgA* developmental defects by the *bcsA*::Tn5Ω951 mutation was due to inactivation of the *bcsA* gene itself and not to polar effects on downstream gene expression. These data also show that the reduction in sporulation observed when *bcsA*⁺ is introduced into the *csgA bcsA* double mutant M3012 is due to complementation of the *bcsA*::Tn5Ω951 mutation rather than to a general undefined inhibitory effect on sporulation efficiency.

Restored expression of a subset of developmentally regulated Tn5*lacZ* fusions in the *bsgA bcsA* mutant. In light of the ability of the *bcsA* mutation to restore fruiting body and spore formation to a BsgA protease-deficient strain, it was of interest to determine whether this was accompanied by restoration of a normal pattern of developmental gene expression. Therefore, the ability of the *bsgA bcsA* mutant to express a collection of nine developmentally induced Tn5*lacZ* fusions was measured and compared to the levels of expression in the *bsgA* mutant and in wild-type cells. The results for the nine *lacZ* fusions are summarized in Table 3, and data for representative *lacZ* fusions are shown in Fig. 4. As has been the case for all developmentally induced *lacZ* fusions reported to date, each of the *lacZ* fusions used in this study was dependent upon the BsgA protease for full levels of developmental expression (14, 29). For three of the nine *lacZ* fusions used in this study, there was significant restoration of developmental expression in the *bsgA bcsA* double mutant compared with that of the *bsgA* mutant. The levels of expression of the Tn5*lacZ* fusions Ω4427 (Fig. 4a) and Ω4411 (data not shown) were restored in a *bsgA bcsA*

FIG. 4. Restored expression of developmentally regulated *lacZ* fusions by the *bsgA bcsA* double mutant during development. The expression of developmental fusions in a *bsgA bcsA* background was compared with expression in the wild type (DZF1) and the *bsgA* mutant (M853). Cells were plated on starvation agar (TPMP) to induce development, and samples were harvested after the indicated time points and frozen. The β-galactosidase specific activity in cell-free sonicates was determined as described in Materials and Methods. Open boxes, wild-type cells; closed boxes, *bsgA* mutant; open triangles, *bsgA bcsA* double mutant. Panel a, Ω4427 fusion; panel b, Ω4414 fusion; panel c, Ω4455 fusion; panel d, Ω4435 fusion.

mutant to levels close to those of the wild type. The level of expression of the $\Omega 4414$ fusion was also restored to a significant degree in the double mutant (fivefold higher than that of the *bsgA* mutant at 48 h), yet expression was temporally delayed compared with that of the *bsgA* mutant (Fig. 4b).

Expression of the six other *lacZ* fusions used in this study was not restored by the *bcsA* mutation in the *bsgA bcsA* double mutant (Table 3). Of the six fusions for which expression was not restored, four ($\Omega 4521$, $\Omega 4455$ [Fig. 4c], $\Omega 4494$, and $\Omega 4273$) are normally expressed earlier than 6 h after initiation of development and two ($\Omega 4435$ [Fig. 4d] and $\Omega 4401$) are normally expressed late in development. These data indicate that the *bcsA* mutation restored expression of a subset of developmental genes to the *bsgA* mutant but did not completely restore the entire developmental program.

Developmental phenotype of the *bcsA* mutant in a wild-type genetic background. To study the phenotype of the Tn5 Ω 951 mutation in an otherwise wild-type (*bsgA*⁺) genetic background, the Tn5 Ω 951 mutation was transduced into the developmentally proficient strains DZF1 and M102. Cells of the resulting *bcsA* mutant strains, M1178 and M1189, respectively, appeared indistinguishable from wild-type cells during vegetative growth with respect to growth rate in rich or minimal medium, colony morphology, and pigmentation. Therefore, the *bcsA* gene is not essential for vegetative growth. In addition, the *bcsA* mutants formed spore-filled fruiting bodies that appeared indistinguishable from those of their respective wild-type parents when plated on starvation agar at high cell density (5×10^7 cells per 10- μ l spot). The number of spores produced by the *bcsA* mutant under these conditions did not differ significantly from that of the wild-type parental strain by either direct counts of refractile, sonication-resistant spores or the number of spores which germinate to form colonies when plated on rich medium (Table 2). Therefore, in contrast to the *bsgA bcsA* double mutant, the *bcsA* mutant develops in a manner that is indistinguishable from that of wild-type cells.

Although the *bcsA* mutant cells were virtually identical to wild-type cells when plated on starvation agar at high cell density (5×10^7 cells per 10- μ l spot), when plated on starvation agar at low cell density (2.5×10^6 cells per 10- μ l spot) (Fig. 5A), the *bcsA* mutant cells completed development in a significantly shorter time than did wild-type cells. As described above, the *bsgA bcsA* double mutant cells also developed faster than wild-type cells when plated at low cell density (Fig. 1, bottom panels). The cloned wild-type *bcsA* gene integrated at the Mx8 *attB* site complemented this aspect of the *bcsA* mutant phenotype and resulted in a strain that was indistinguishable from that of the wild-type cells when plated on starvation agar at low cell density (data not shown). In addition, in a strain containing two copies of the wild-type *bcsA* gene, one at the normal chromosomal site and the other a cloned copy at the Mx8 *attB* site, development was temporally delayed at low cell density compared to that of wild-type cells (data not shown). These findings suggest that the *bcsA* gene product has an inhibitory effect on development when wild-type cells are plated on starvation agar at low cell density and that the inhibitory effect increases with increasing *bcsA* copy number.

***bcsA* mutants express elevated levels of a developmentally induced, cell-density-regulated gene during vegetative growth**

FIG. 5. Effects of cell density on aspects of the *bcsA* mutant phenotype. (A) Rapid development of a *bcsA* mutant plated on starvation agar at low cell density. Development was initiated by plating cells on CF agar at low density (2.5×10^6 cells per 10- μ l spot). Spots were photographed after incubation for 3 days at 30°C. (Panel a) DZF1 (wild type). (Panel b) M1178 (DZF1 *bcsA*::Tn5 Ω 951). (B) Cell-density-dependent expression of fusion $\Omega 4427$ by the *bcsA* mutant, growing in rich medium. Strains containing the Tn5 Ω 951 fusion in a wild-type genetic background (solid dark bars) or in a *bcsA* mutant background (lightly shaded bars) were grown in CTT broth. Samples were harvested at various times, and the β -galactosidase specific activity was determined. Veg., logarithmically growing cells at a culture density of 2.5×10^8 cells per ml; E.S., early stationary phase, 2 h after culture reached maximum cell density; L.S., late stationary phase, 12 h after culture reached maximum cell density.

and stationary phase. The expression of a set of twelve developmentally induced Tn5 Ω 951 fusions was examined to identify genes for which expression in a *bcsA* mutant may become dysregulated during growth or stationary phase in rich medium. Such a phenotypic effect has precedence in the behavior of a previously reported *bsgA* suppressor conferred by mutations in *spdR* (19). Each fusion was transduced into the wild-type and *bcsA* mutant strains, and the levels of β -galactosidase expression were compared at various stages of growth in rich medium. The level of β -galactosidase expression was not altered by the *bcsA* mutation for 11 of 12 of the fusions. However, the fusion $\Omega 4427$ was overexpressed five- and eightfold during logarithmic growth and stationary phase, respectively, by the *bcsA* mutant compared with that of otherwise isogenic wild-type cells (Fig. 5B). These results indicate that *bcsA* altered the vegetative expression of at least one developmentally regulated gene. In light of the influence of cell density on the developmental phenotype of the *bcsA* mutant and the finding that the *bcsA* mutations dramatically alter the regulation of the $\Omega 4427$ fusion, it may be noteworthy that the developmental expression of the $\Omega 4427$ fusion itself has been reported to be cell density dependent.

DISCUSSION

***bcsA* mutations bypass the developmental requirement for B and C signaling.** This work reports the identification of a locus (*bcsA*) in which mutations have the novel phenotype of bypassing the requirements for both B and C signaling, thereby restoring the ability of both *bsgA* and *csgA* mutants to form fruiting bodies and viable spores. Although the *bcsA* mutants bypassed the requirements for B and C signaling, the requirement for A signaling was not affected.

Earlier studies have shown that *asg*, *bsgA*, and *csgA* mutations block developmental gene expression at distinct temporal stages of the developmental pathway (14, 24, 29, 36). Both *asg* and *bsgA* mutations affect development at a very early stage, reducing or abolishing expression of nearly all known developmental markers, including markers normally expressed within the initial few hours of development. In contrast, *csgA* mutants appear to be blocked at a considerably later developmental stage, resulting in reduced or abolished transcription of markers that begin to be expressed after approximately 6 h of development.

The finding that mutations in *bcsA* do not eliminate the requirement for A signaling indicates that these mutations do not simply eliminate all of the signaling requirements during the early portions of the developmental program. These observations also suggest the novel and exciting possibility that the B and C signaling pathways, although genetically quite distinct, share a common regulatory pathway which is influenced by BcsA but is independent of the A signaling pathway.

Early in the starvation-induced initiation of development, cells undergo a stringent response which is both required and sufficient for initiation of at least early events in the developmental program (38, 39, 46). Crawford and Shimkets (9) have reported evidence that the *csgA* gene product may play a role early in development by amplifying and prolonging the stringent response into later times of development. It will be of interest to determine whether *bcsA* and *bsgA* also affect the regulation of the stringent response, thereby providing a unifying mechanistic relationship.

The data presented here show that *bcsA* restores expression of a discrete subset of developmentally induced *lacZ* fusions to the *bsgA* mutant but does not restore a wild-type pattern of developmental gene expression. Of the nine developmentally induced *lacZ* fusions that were used in these studies, the expression of three was restored to levels significantly higher than that of the *bsgA* mutant alone during development; expression of the other six was unaffected. It is noteworthy that the three *lacZ* fusions with restored expression had expression times of 6 h or more; transcription of the four *lacZ* fusions with expression times less than 6 h was not restored. However, mutations in *bcsA* do not bypass all early portions of the developmental program. For example, the *bcsA* mutants retain the requirement for A signaling. The *bsgA bcsA* double mutant restores nearly wild-type levels of expression to at least one developmentally induced gene ($\Omega 4411$) that is normally expressed prior to the requirement for C signaling, and conversely, not all late C-signal-dependent gene expression (e.g., that of $\Omega 4435$ and $\Omega 4401$) is restored.

None of the three restored fusions are themselves required for development under laboratory conditions (30). Instead,

they are likely to be representatives of a regulatory class which includes additional gene(s) that play a key regulatory role during development. It is likely that expression of such genes by the *bcsA* mutant is sufficient to allow the B- and C-signaling mutant cells to bypass certain portions of the developmental program and to reenter the program at a later temporal stage, at which time gene expression is not directly dependent on these signals.

Relationship of *bcsA* to other suppressors of *bsgA*. The *spdR* gene is the only other well-characterized gene in which mutations enable *M. xanthus* cells to develop in the absence of the BsgA protease (19). The *spdR* gene encodes a putative response regulator with homology to the NtrC family. However, our data suggest that the *bcsA* gene does not function in the same pathway as the *spdR* gene. The *bcsA* mutant is able to bypass the requirement for C signaling yet does not bypass the requirement for A signaling. Conversely, the *spdR* mutant bypasses the requirement for A signaling yet does not bypass the requirement for C signaling (51). Additionally, the *bcsA* mutant exhibits elevated levels of expression of the fusion $\Omega 4427$ during vegetative and logarithmic growth, an effect not observed with the *spdR* mutant (18). The *spdR* mutant overexpresses the fusion $\Omega 4273$ (*tps*) in vegetative growth and exhibits reduced expression levels of the fusion $\Omega 4521$ in development (18), two characteristics not observed with the *bcsA* mutant. Based on the nonoverlapping phenotypes of the *bcsA* and *spdR* mutants, it is unlikely that the *bcsA* and *spdR* genes function in the same regulatory pathway. It is therefore possible that the BsgA protease-controlled pathway of development branches early, with *spdR* occupying one branch which interacts with A signaling and the *bcsA* locus being present in a separate parallel branch which interacts with C signaling.

BcsA may act to inhibit progress through the developmental program. Several lines of evidence suggest that the *bcsA* gene product normally plays a role in inhibiting the progression of cells through the developmental program. Each of the phenotypic properties associated with loss of BcsA function is consistent with its inhibitory role, including the following: (1) bypass of the developmental requirement for two distinct signaling steps, (2) significantly faster development than that of wild-type cells when plated on starvation agar at low cell density (whereas additional copies of *bcsA* result in a delayed development program), and (3) inappropriate expression of at least one developmentally regulated gene during vegetative growth.

In the first line of evidence for an inhibitory role for the *bcsA* gene product, one possible interpretation of the bypass of B and C signaling is that the *bcsA* product functions to inhibit development, which is typically overcome by the concerted effect of B and C signaling. However, in a *bcsA* mutant, and thus in the absence of inhibition by the *bcsA* product, development becomes partially independent of the B and C signals. The finding that the A signal requirement does not appear to be affected suggests that BcsA-mediated inhibition is a specific result of interaction with a pathway that includes the B and C signals.

Second, although the kinetics of development of the *bcsA* mutant are comparable to those of the wild type at high cell density, the *bcsA* gene product inhibits development at low cell density and may do so in a dosage-dependent manner. It is not

obvious why this inhibitory effect of *bcsA* should be most apparent at low cell density. Yet it should be emphasized that the effects of *bcsA* are not restricted to development at low cell density. For example, a *bcsA* mutation also relieves the requirement for both B and C signaling when cells are plated at high cell density.

A third line of evidence suggesting that *bcsA* functions in an inhibitory manner is that vegetative expression of the developmentally induced $\Omega 4427$ *lacZ* fusion appears to be inhibited in the presence of the *bcsA* gene product, since both *bcsA* and *bsgA bcsA* mutants overexpress the $\Omega 4427$ fusion during vegetative growth. Thus, the *bcsA* gene product appears to contribute to the inhibition of expression of certain developmentally induced genes during vegetative growth. An intriguing possibility is that the vegetative expression of genes such as $\Omega 4427$ in the *bcsA* mutant provides a mechanism for *bcsA* mutants to enter the developmental pathway in a manner that is independent of the B and C signals. If a subset of the developmentally induced genes is expressed in *bcsA* mutants during vegetative growth, then expression of those genes in vegetative growth may provide the ability to induce portions of the developmental program upon starvation in either the *bsgA bcsA* or *csgA bcsA* double mutants. In light of the correlation between cell density and an exacerbation of the *bcsA* mutant phenotype, it is particularly interesting that the expression of the $\Omega 4427$ fusion has been reported to be cell density regulated in wild-type cells in a manner that is independent of A signaling. Presently, the A signal is the only quorum-sensing molecule identified in *M. xanthus*. Future experiments will be needed to address both how the $\Omega 4427$ fusion responds to cell density independently of the A signal and how the BcsA protein functions to inhibit the expression of the $\Omega 4427$ fusion.

The *bcsA* product has similarity to flavin-containing monooxygenases. The Tn5 Ω 951 insertion was mapped to a gene, *bcsA*, which is predicted to encode a protein with significant similarity to a recently identified group of related flavin-containing monooxygenases, designated StyA. Additional studies are necessary to determine whether the BcsA protein exhibits monooxygenase activity and what the role of such an enzymatic activity may be in *M. xanthus* development. We have also observed that the *bcsA* mutant develops on medium containing nutrient levels high enough to block development in wild-type cells (Cusick and Gill, unpublished observations). This finding would more likely implicate the *bcsA* product in the sensing of the response of the cell to nutritional deprivation. Interestingly, the CsgA protein appears to function to influence the stringent response and has homology to short-chain alcohol dehydrogenases. How the CsgA dehydrogenase homolog and the BcsA monooxygenase homolog act to enhance and inhibit development, respectively, remains an exciting area of continuing research.

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