Characterization of \textit{bcsA} Mutations That Bypass Two Distinct Signaling Requirements for \textit{Myxococcus xanthus} Development

John K. Cusick,† Elizabeth Hager, and Ronald E. Gill*

Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received 3 December 2001/Accepted 18 June 2002

The BsgA protease is required for starvation-induced development in \textit{Myxococcus xanthus}. Bypass suppressors of a \textit{bsgA} 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 \textit{bsgA} mutant and was capable of forming fruiting bodies and viable spores in the absence of the BsgA protease. The Tn5 insertion was localized to a gene, \textit{bcsA}, that encodes a protein that has significant amino acid similarity to a group of recently described flavin-containing monoxygenases involved in styrene catabolism. Mutations in \textit{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 \textit{bcsA} mutation was accompanied by restored expression of a subset of developmentally induced lacZ fusions to the BsgA protease-deficient strain. \textit{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 (\textit{O14427}), suggesting that the \textit{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 \textit{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.

\textit{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 \textit{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 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 \textit{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 Tn5lac transposon (31). Each Tn5lac 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 Tn5lac 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 \textit{bsgA}. (15, 35). The \textit{bsgA} mutants do not form fruiting bodies or spores and so by morphological criteria appear to be blocked very...
M. xanthus plays a central role in initiating the developmental program. The BsgA protease substrate(s) predicted to encode a protein that shares a high degree of 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, Tn5132, which is localized to a gene designated bcsA. The BsgA protease substrate(s) critical for regulation of developmental gene expression in M. xanthus has not yet been identified.

TABLE 1. Myxococcus xanthus strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source and/or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZF1</td>
<td>Developmentally proficient genetic background used for certain strain constructions; grows dispersed in liquid culture due to an S-motility mutation in pilQ</td>
<td>D. Zusman; 41 and 52</td>
</tr>
<tr>
<td>M853</td>
<td>DZF1 bcsA330</td>
<td>19</td>
</tr>
<tr>
<td>M951</td>
<td>M853 bcsA::Tn5132</td>
<td>This study</td>
</tr>
<tr>
<td>M972</td>
<td>M853 bcsA::[Tn5132::Tn5] Tn5132</td>
<td>This study</td>
</tr>
<tr>
<td>M1178</td>
<td>DZF1 bcsA::Tn5132</td>
<td>This study</td>
</tr>
<tr>
<td>M3009</td>
<td>DZF1 bcsA::Tn5132</td>
<td>This study</td>
</tr>
<tr>
<td>DK101</td>
<td>Developmentally proficient genetic background used for certain strain constructions; grows dispersed in liquid culture due to an S-motility mutation, sglA1</td>
<td>D. Kaiser</td>
</tr>
<tr>
<td>DK731</td>
<td>M101 csgA731</td>
<td>D. Kaiser; 17</td>
</tr>
<tr>
<td>M3011</td>
<td>M101 csgA731 bcsA::Tn5132</td>
<td>This study</td>
</tr>
<tr>
<td>M3012</td>
<td>M101 csgA731 bcsA::Tn5132 bcsA::Tn5132</td>
<td>This study</td>
</tr>
<tr>
<td>M3013</td>
<td>M101 bcsA::Tn5132</td>
<td>This study</td>
</tr>
<tr>
<td>DK1622</td>
<td>Developmentally proficient genetic background used for certain strain constructions; fully motile</td>
<td>23</td>
</tr>
<tr>
<td>LS523</td>
<td>DK1622csgA205</td>
<td>45</td>
</tr>
<tr>
<td>M1187</td>
<td>LS523 bcsA1187; bcsA1187 was introduced by integration of a plasmid containing an internal bcsA restriction fragment</td>
<td>This study</td>
</tr>
<tr>
<td>M252</td>
<td>DK1622bcsA330</td>
<td>15</td>
</tr>
<tr>
<td>M1188</td>
<td>M252bcsA1187; bcsA1187 was introduced as in M1187</td>
<td>This study</td>
</tr>
<tr>
<td>M1189</td>
<td>DK1622bcsA::Tn5132</td>
<td>This study</td>
</tr>
</tbody>
</table>

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.

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 Tn5lac transposon (28), independent Tn5lac insertions are designated by an omega number (e.g., Tn5lacM04521 or simply D4521). For strain constructions, individual Tn5lac insertions were transferred by generalized transduction and selected for kanamycin resistance.

The bcsA330(Tn5) allele contains a Tn10-derived BglII fragment of the tetracycline resistance gene that was cloned into the unique BglII site of the M. xanthus bsgA gene (19). Strain M951 was created by Tn5 mutation of the bcsA330 mutant strain M853, using bacteriophage P1::Tn5 (32). Strain M972 is a derivative of M951 in which the kanamycin resistance of Tn5132 has been replaced with trimethoprim resistance. The Tn5 insertion in M972 remains in precisely the same location as Tn5132, as determined by Southern blot analysis, and confers the same phenotype as the original Tn5132 mutation. Replacement of kanamycin resistance by trimethoprim resistance was performed using P1::[Tn5132] (obtained from L. Shimkets) as previously described (2). Similarly, strain M3009 is a derivative of M951 in which the kanamycin resistance of Tn5132 has been replaced with tetracycline resistance, using P1::Tn5-132 as previously described (2).

The myxophage Mx8 left lrm ts18s27 (6) was used for strain constructions by using generalized transduction. The coliphage P1ev100Cm (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 TPM agar (TPM supplemented with 1% pyruvate and 1.8% agar).

Recombinant genetic methods. Chromosomal DNA purification, transformation, 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 Kashefi 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 Tn5132 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 μg/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’ GTTCCGTTCAGGACGCTA and complementary to Tn5 nucleotides 18 to 37 and 5’ GTGATCTCTGCGCATGTC (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 Tn51 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 Tn5D951 insertion into the csgA205 mutant strain (LS23) 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 LS23 and a bcsA 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-tn5-132D951 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-tn5-132D951 double-mutant strain M3011.

**Complementation of bcsA in a bsgA bcsA mutant.** The XhoI and SrfI 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 Ms2 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 assay.** A single, isolated colony was grown in CTT broth to 5 to 105 cells per ml, washed, and resuspended 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 suspensions 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 bsgA 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 bsgA null mutant. The bsgA330 mutant M853 was mutagenized with Tn5, followed by screening of individual Tn5-containing (Km') 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 spuruous mutation that may have occurred during the selection process. The Tn5 insertion from the M951 donor strain (designated Tn51951) was transduced into the parental bsgA330 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 Tn51951 insertion at its original position and the presence of the bsgA330 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 right, center 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 x 107 cells per 10-μl spot). In contrast, the bsgA 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 x 106 cells per 10-μl spot). After 3 days of development at low cell density (Fig. 1, center row), strain...
M951 had formed dark, spore-filled fruiting bodies while the wild-type strain had produced only translucent aggregates that were devoid of refractive 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 refractive 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 × 10⁷ cells per 10-μl 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 viable 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::Tn5951 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 T5951 locus

Inverse PCR was used as described in Materials and Methods to amplify a DNA fragment containing the Tn5951-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 Tn5951 insertion site and from which a hybridizing 1.5-kb SphI fragment and an overlapping 2.0-kb BglII fragment were cloned. The two fragments were sequenced, yielding approximately 3 kb of contiguous DNA sequence in the vicinity of the Tn5951 insertion (Fig. 2). The precise position of the Tn5 insertion in strain M951 was determined by sequencing the Tn5951 inverse PCR product. From these data, we determined that the Tn5951 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 CA04000.1 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-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Strain description</th>
<th>% of wild-type numbers of viable sporesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZF1</td>
<td>Developmentally proficient</td>
<td>100</td>
</tr>
<tr>
<td>M853</td>
<td>DZF1 bsgA4338</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>M951</td>
<td>DZF1 bsgA bcsA::Tn5951</td>
<td>93</td>
</tr>
<tr>
<td>M1187</td>
<td>DZF1 bcsA::Tn5951</td>
<td>93</td>
</tr>
<tr>
<td>M3010</td>
<td>DZF1 bsgA bcsA::Tn5951/bcsA+</td>
<td>0.23</td>
</tr>
<tr>
<td>DK1622</td>
<td>Wild-type</td>
<td>100</td>
</tr>
<tr>
<td>LS523</td>
<td>DK1622 csgA4205</td>
<td>0.16</td>
</tr>
<tr>
<td>M1187</td>
<td>DK1622 csgA4205 csgA41187</td>
<td>14</td>
</tr>
<tr>
<td>DK101</td>
<td>Developmentally proficient</td>
<td>100</td>
</tr>
<tr>
<td>DK731</td>
<td>DK101 csgA731</td>
<td>0.09</td>
</tr>
<tr>
<td>M3011</td>
<td>DK101 csgA731::Tn5::132f951</td>
<td>12</td>
</tr>
<tr>
<td>M3012</td>
<td>DK101 csgA731 bcsA::Tn5::132f951/bcsA+</td>
<td>0.14</td>
</tr>
<tr>
<td>M3013</td>
<td>DK101 bcsA+::bcsA+</td>
<td>96</td>
</tr>
</tbody>
</table>

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.
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 region, 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 βββ 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).

FIG. 2. Map of the region surrounding the Tn5951 insertion and complementation of the bcsA::Tn5951 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 Tn5951 insertion located within the amino-terminal portion of a bcsA is indicated (arrowhead). Restriction enzyme sites are shown as follows: S, SstI; X, XhoI, B, BglII. (B) Complementation of the suppressor phenotype of the bcsA::Tn5951 mutant. Cells were plated on CF medium to induce development and photographed after 6 days at 30°C. (Panel a) M853 (parental bcsA mutant). (Panel b) M972 (bcsA Tn5951 [Ty]). (Panel c) containing the SsrI fragment cloned into pREG1962 and integrated at the Mx8 attB site. (Panel d) M972 (containing the XhoI fragment cloned into pREG1962 and integrated at the Mx8 attB site).

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

<table>
<thead>
<tr>
<th>Fusion</th>
<th>Expression time (h)</th>
<th>B- or C-signaling requirement</th>
<th>Restored expression in bsgA bcsA double mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ω4521</td>
<td>2</td>
<td>B</td>
<td>−</td>
</tr>
<tr>
<td>Ω4455</td>
<td>2</td>
<td>B</td>
<td>−</td>
</tr>
<tr>
<td>Ω4494</td>
<td>2</td>
<td>B</td>
<td>−</td>
</tr>
<tr>
<td>Ω2473</td>
<td>5</td>
<td>B</td>
<td>−</td>
</tr>
<tr>
<td>Ω4411</td>
<td>6</td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td>Ω4427</td>
<td>7</td>
<td>B, C</td>
<td>+</td>
</tr>
<tr>
<td>Ω4414</td>
<td>12</td>
<td>B, C</td>
<td>+</td>
</tr>
<tr>
<td>Ω4435</td>
<td>25</td>
<td>B, C</td>
<td>−</td>
</tr>
<tr>
<td>Ω4401</td>
<td>25</td>
<td>B, C</td>
<td>−</td>
</tr>
</tbody>
</table>

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 monoxygenase 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::Tn5fl951 mutation by the cloned bcsA + gene. To demonstrate that the phenotype attributed to the Tn5fl951 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::Tn5fl951[Tmp]; 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 Tn5fl951 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 SsrI 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::Tn5fl951 mutation in a csgA mutant. As shown in Table 2, introduction of the cloned wild-type bcsA gene into the csgA731 bcsA::Tn5fl951 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-
mutant to levels close to those of the wild type. The level of expression of the Ω4414 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 (Ω4521, Ω4455 [Fig. 4c], Ω4494, and Ω4273) are normally expressed earlier than 6 h after initiation of development and two (Ω4435 [Fig. 4d] and Ω4401) 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 Tn5Ω951 mutation in an otherwise wild-type (bsgA−) genetic background, the Tn5Ω951 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 x 10⁷ cells per 10-μl 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 x 10⁷ cells per 10-μl spot), when plated on starvation agar at low cell density (2.5 x 10⁶ cells per 10-μl 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 and stationary phase.** The expression of a set of twelve developmentally induced Tn5lacZ 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 Ω4427 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 Ω4427 fusion, it may be noteworthy that the developmental expression of the Ω4427 fusion itself has been reported to be cell density dependent.
DISCUSSION

*bcxA* mutations bypass the developmental requirement for B and C signaling. This work reports the identification of a locus (*bcxA*) 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 *bcxA* 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 *bcxA* 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 *bcxA* and *bsgA* also affect the regulation of the stringent response, thereby providing a unifying mechanistic relationship.

The data presented here show that *bcxA* 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 *bcxA* do not bypass all early portions of the developmental program. For example, the *bcxA* 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 (Ω4411) 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 Ω4435 and Ω4401) 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 *bcxA* 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 *bcxA* 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 *bcxA* gene does not function in the same pathway as the *spdR* gene. The *bcxA* 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 *bcxA* mutant exhibits elevated levels of expression of the fusion Ω4427 during vegetative and logarithmic growth, an effect not observed with the *spdR* mutant (18). The *spdR* mutant overexpresses the fusion Ω4273 (Ωs) in vegetative growth and exhibits reduced expression levels of the fusion Ω4521 in development (18), two characteristics not observed with the *bcxA* mutant. Based on the nonoverlapping phenotypes of the *bcxA* and *spdR* mutants, it is unlikely that the *bcxA* 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 *bcxA* 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 *bcxA* 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 *bcxA* 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 *bcxA* gene product, one possible interpretation of the bypass of B and C signaling is that the *bcxA* product functions to inhibit development, which is typically overcome by the concerted effect of B and C signaling. However, in a *bcxA* mutant, and thus in the absence of inhibition by the *bcxA* 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 *bcxA* mutant are comparable to those of the wild type at high cell density, the *bcxA* 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 Ω4427 lacZ fusion appears to be inhibited in the presence of the bcsA gene product, since both bcsA4427 and bsgA bcsA mutants overexpress the Ω4427 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 Ω4427 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 Ω4427 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 Ω4427 fusion responds to cell density independently of the A signal and how the BcsA protein functions to inhibit the expression of the Ω4427 fusion.

The bcsA product has similarity to flavin-containing monoxygenases. The Tn51951 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 monoxygenases, designated SfyA. 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.

ACKNOWLEDGMENTS

We thank Martin Pato for his critical evaluation of these experiments and careful reading of the manuscript. This work was funded by awards to R.E.G. from the National Institutes of Health (GM31900) and National Science Foundation (MCB-9631365).

REFERENCES


