csgA expression entrains *Myxococcus xanthus* development

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The developmental cycle of the myxobacterium *Myxococcus xanthus* consists of three partially overlapping morphological stages referred to as rippling, fruiting body formation, and sporulation, all of which are absent in *csgA* null mutants. The CsgA gene product is an extracellular protein, referred to as the C signal, which is essential for developmental cell-cell interactions. *csgA* expression increases throughout development, reaching its peak during sporulation. CsgA was made limiting for development by constructing nested deletions upstream from the *csgA* gene, which resulted in reduced *csgA* expression. Successively larger deletions resulted in termination of development at earlier and earlier stages, with rippling requiring ~20% maximum *csgA* expression, fruiting body formation requiring ~30% expression, and sporulation requiring 82% expression. Conversely, artificial induction of *csgA* also induced development provided nutrients were limiting. These results suggest that steady increases in CsgA over the course of development entrain the natural sequence of morphological events. The *csgA* upstream region appears to process information concerning the levels of nutrients, peptidoglycan components, and the B signal. In the absence of nutrients, a region extending 400 bp upstream from the start site of transcription was necessary for development and maximal *csgA* expression. In the presence of low levels of nutrients, a region extending ~930 bp upstream was essential for the same tasks. It appears that the upstream region extending from -400 to -930 stimulates *csgA* expression in the presence of excess carbon, nitrogen, and phosphate, thereby allowing development to go to completion.

[Key Words: *Myxococcus xanthus*; fruiting body formation; morphogen; developmental timer; gene expression]

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The myxobacterium *Myxococcus xanthus* forms fruiting bodies in response to starvation. Developing cells proceed through a series of partially overlapping but morphologically distinct stages known as rippling, aggregation, and sporulation (for review, see Shimkets 1990). During vegetative growth and the earliest developmental stage, the cells arrange themselves in a series of equidistant ridges known as ripples that move processively to give the appearance of pulsating waves (Reichenbach 1965; Shimkets and Kaiser 1982). Rippling is not essential for fruiting body formation because nonrippling mutants can still form fruiting bodies. During the second morphological stage of development, tens of thousands of cells glide to an aggregation center where they form a hemispherical fruiting body that is 100 μ m high. The rod-shaped vegetative cells differentiate into spherical, dormant myxospores inside the fruiting body during the third stage.

The temporal and spatial coordination of development

is regulated by intercellular communication between *M. xanthus* cells. The intercellular signal most extensively studied is the C signal, which is the protein product of the *csgA* gene (Hagen and Shimkets 1990; Kim and Kaiser 1990a,b; Shimkets and Rafiee 1990). Strains containing a null *csgA* mutation do not ripple, aggregate, or sporulate (Shimkets et al. 1983; Shimkets and Asher 1988) and cease developmental gene expression after ~6 hr (Kroos and Kaiser 1987). The CsgA gene product appears to be extracellular and is associated with the cell surface and extracellular matrix (Shimkets and Rafiee 1990). Kim and Kaiser (1991) added the purified CsgA protein to a *csgA* null mutant and noticed that aggregation was induced at a lower concentration than sporulation.

The idea that aggregation and sporulation are induced at different CsgA concentrations was examined by manipulating the *csgA* upstream regulatory region to control CsgA production in vivo. Our results suggest that rippling, aggregation, and sporulation have different thresholds for induction by CsgA. Furthermore, *csgA* expression appears to be controlled by carbon, nitrogen, and phosphate levels, peptidoglycan availability, and the concentration of the B signal.

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Results

csgA transcriptional start site

The transcriptional start site for the *csgA* gene was identified by primer extension using RNA isolated from rippling and aggregating cells, 18 hr after the initiation of development. An oligonucleotide complementary to the mRNA was used for reverse transcriptase primer extension and produced one major product and two minor products (Fig. 1). The same major primer extension product was observed with RNA prepared from vegetative and 96-hr developing cells (data not shown). The nucleotide sequence of the homologous *csgA* DNA was determined using the same primer with the dideoxy chain-termination method and is shown next to the primer extension products (Fig. 1). Upstream from this initiation site is a putative promoter with –35- and –10-bp sequences that are similar to the *Escherichia coli* σ^{75} consensus –35 (TTGACA) and –10(TATAAT) sequences (Helmann and Chamberlin 1988). A similar transcriptional start site was observed using the S1 nuclease method (data not shown), and these results are also consistent with an 800-nucleotide *csgA* mRNA observed by Northern blotting (Hagen and Shimkets 1990).

Effect of *CsgA* limitation

To determine which upstream sequences are essential in *cis* for *csgA* transcription, a series of nested deletions was constructed across the upstream region and fused with the *lacZ* reporter gene in an integrating vector (Fig. 2). Each of the constructs was transduced into LS523 cells, which contain a *csgA* null mutation, where they integrated into the chromosome at the phage Mx8 attachment site (Shimkets and Asher 1988). The cells were allowed to develop on CF agar and assayed for β -galactosidase activity at 12- to 24-hr intervals, and the peak β -galactosidase specific activity of each of the seven deletion strains was compared with that of the *csgA* mutant containing pLJS94, which contains 2400 bp upstream from the transcriptional start site (Fig. 2). As the upstream region decreased in length, the peak level of β -galactosidase also decreased, with maximal β -galactosidase accumulation requiring an upstream regulatory region of ~930 bp. This large region is required in *cis* because an intact copy of this upstream region is located next to the mutant *csgA* allele elsewhere on the chromosome. Deletion of the entire upstream regulatory region in pLJS51, including the putative promoter and transcriptional start site, virtually eliminated expression.

To determine the effect that reduced *csgA* expression has on development, the same nested deletions were placed upstream of the *csgA* structural gene and transduced into LS523. The cells were observed for ripples, fruiting bodies, and spores on CF agar. Rippling was qualitatively assessed 12 and 24 hr later and was observed in all strains except those containing pLJS51, which lacks the putative promoter and transcriptional start site. Therefore, rippling requires an upstream regulatory re-

Figure 1. The *csgA* transcriptional start site determined by primer extension. Total RNA was harvested 18 hr after initiation of development. Synthetic oligonucleotide complementary to the *csgA* RNA (5'-dGACCGGTACACACGTTGGTG-3') was used as a primer for both reverse transcriptase primer extension (right lane) and nucleotide sequencing of *csgA* DNA by the dideoxy chain-termination method (middle lanes labeled GATC). The DNA sequence of the putative promoter region is given at left. The bold T residue indicates the transcriptional start site. Boxes refer to the –10 and –35 regions of the promoter.

gion of <266 bp. At the low cell densities used in this experiment the fruiting bodies had nearly perfect hemispherical shapes, permitting a quantitative assessment of the extent of aggregation based on the combined volumes of the fruiting bodies. The total fruiting body volume was calculated from the number of fruiting bodies per plate and their average diameters. The highest proportion of cells were located in fruiting bodies with se-

Figure 2. Physical maps of the *csgA* gene fragments used in this study and comparisons of the relative levels of aggregation, sporulation, and β -galactosidase activity of LS523 (*csgA*) strains containing these constructs. Plasmids are listed in pairs that have identical upstream regulatory regions, with the plasmid at the left containing a functional *csgA* structural gene to assess developmental competence and the one at the right containing a *csgA-lacZ* transcriptional fusion to assess *csgA* expression. The precise sizes of the upstream regions were determined by sequencing the DNA to locate the restriction sites used in the cloning and are listed below relative to the transcriptional start site: pLJS80/pLJS94, -2400 bp; pLJS93/pLI212, -930 bp; pLJS92/pLI211, -729 bp; pLJS91/pLI210, -676 bp; pLJS58/pLI227, -400 bp; pLJS63/pLI223, -336 bp; pLI226/pLI217, -266 bp; pLJS51/pLI225, +41 bp. β -Galactosidase activities (solid bars) are given as the peak developmental activities relative to the peak activity of LS523 containing pLJS94.

Aggregation was assayed after 2 days by calculating the total volume of the fruiting bodies relative to that of LS523 containing pLJS80 (hatched bars). Spore number was determined after 5 days relative to that of LS523 containing pLJS80 (stippled bars). The results are the average of two experiments with the variation in the relative activities between experiments <10%.

quences extending at least 400 bp upstream from the transcriptional start site (Fig. 2). Sporulation was assayed by counting the number of sonication-resistant spores under a phase-contrast microscope after 120 hr of development. Maximum spore yields required ~930 bp upstream from the transcriptional start site (Fig. 2). Cells containing constructs that were too short to permit sporulation, such as pLJS58, had the unusual property of dispersing from the nascent fruiting bodies at ~96 hr. By comparing the rippling, aggregation, and sporulation phenotypes of each strain a general pattern emerged: As more of the upstream sequences were removed, the cells progressed through less and less of the developmental pathway. Because the only differences between these strains is the length of the upstream regions, and consequently the amount of *csgA* expression, premature termination of development in the deletion mutants is the result of limitation of CsgA.

The relationship between *csgA* expression and developmental progress was examined in *csgA*⁺ cells, following transduction of DK1622 with pLJS94, which contains the full-length *csgA* upstream region fused to *lacZ*, and assessing *csgA* expression throughout development. β -Galactosidase activity increased throughout development, reaching its peak during sporulation at ~72 hr [Figs. 4 and 6 (below) provide typical examples]. The range of *csgA* expression observed during each developmental stage is denoted by the labeled, open boxes in Figure 3. Each morphological stage begins at a specific and unique level of *csgA* expression. In the graph the extent of rippling, aggregation, and sporulation by cells containing each deletion construct from Figure 2 was

replotted against the maximum amount of *csgA* expression observed with that upstream region. The pLJS51/pLI225 plasmid pair lacked the *csgA* promoter, did not induce any of the morphological signs of development, and exhibited little *csgA* expression. The pLI226/pLI217 plasmids, which contained the next largest upstream regulatory region, exhibited normal rippling, ~30% wild-type aggregation, no sporulation, and ~20% maximum *csgA* expression. From these results it appears as if rippling is induced when *csgA* expression exceeds 20% of maximum, in good agreement with the appearance of ripples at a similar *csgA* expression level with wild-type cells as shown in the labeled, open boxes above the graph. The number of cells in fruiting bodies increased with increasing *csgA* expression until ~30% expression with pLJS58, again in good agreement with the level of expression observed during wild-type aggregation. Spore number reached its peak at ~82% expression with pLJS93, similar to expression levels observed during wild-type sporulation. In each case, *csgA* expression at the beginning of a particular developmental stage in wild-type cells was slightly higher than *csgA* expression of a deletion strain that ceased development prior to that stage. Because each developmental stage appears to have a different threshold for induction by CsgA, stage-specific increases in *csgA* expression could entrain the morphological stages in their correct temporal order.

Artificial induction of *csgA* expression

Another way to test the hypothesis that developmental progress is determined by the extent of *csgA* expression

Figure 3. Comparison of wild-type *csgA* expression during each developmental stage with the terminal developmental phenotypes of the deletion mutants. To determine the range of *csgA* expression observed during rippling, aggregation, and sporulation of wild-type cells (DK1622) containing pLJS94, cells were allowed to develop on CF agar and assayed for β -galactosidase at 12- to 24-hr intervals. The range of *csgA* expression observed during each morphological stage of development is given by the labeled open bars above the graph. For example, rippling was first observed when wild-type cells achieved 15% maximum *csgA* expression and continued until ~60% expression. Below the bars the rippling (○), aggregation (□), and sporulation (■) phenotypes of the deletion strains are plotted against the maximum level of *csgA* expression observed with that pair of strains (data are replotted from Fig. 2). The plasmid pairs listed along the x-axis contain identical upstream regulatory regions, with the upper plasmid containing the *csgA* structural gene to assess developmental progress (relative to that of LS523 containing pLJS80) and the lower plasmid containing the *lacZ* structural gene to assess *csgA* expression (relative to that of LS523 containing pLJS94). For example, pLI226 induced normal rippling, 30% aggregation, and <1% sporulation (x-axis), whereas pLI217 induced 20% of maximum *csgA* expression (y-axis). It is significant that the transition from the nonrippling phenotype of strains containing pLJS51 to the rippling phenotype of strains containing pLI226 is accompanied by an increase in *csgA* expression to a level in which rippling is observed in wild-type cells. Similarly, the transition from the nonaggregating phenotype of strains containing pLJS51 to the aggregating phenotype of strains containing pLJS58 is accompanied by an increase in *csgA* expression to a level produced by aggregating wild-type cells. In addition, the transition from the nonsporulating phenotype of pLJS58 to the sporulating phenotype of pLJS80 is accompanied by an increase in *csgA* expression to levels observed during wild-type sporulation.

involves placing the *csgA* structural gene under the control of a different promoter and determining whether the cells develop normally upon artificial induction of *csgA* expression. The *carQRS* promoter (Letouvet-Pawlak et al. 1990) was ligated in the proper orientation to the *KpnI* site of the *csgA* regulatory region of pLJS58 to create a *carQRS-csgA* transcriptional fusion in pBUL31. LS523 cells containing pBUL31 sporulated as efficiently as cells containing pLJS80, in marked contrast to cells containing pLJS58, which formed few spores (Table 1). To determine whether these sporulation differences were the result of differences in the level of *csgA* expression, *carQRS* promoter expression was measured using pDAH217, which contains a *carQRS-lacZ* fusion. Expression of the *carQRS* promoter under these conditions was comparable to that of the full-length *csgA* upstream region in pLJS94 and significantly higher than that of pLI227 (Table 1). Therefore, artificial induction of *csgA* transcription results in marked stimulation of development under normal developmental conditions.

It is perhaps fortuitous that the *carQRS* promoter effectively substituted for the *csgA* upstream region, as the two regulatory regions induced similar rates of increase in β -galactosidase during development. Strong and rapid induction of *csgA* transcription might disrupt the normal pattern of development and induce sporulation prior to the completion of aggregation. The *carQRS* promoter can be induced ~50-fold with white light illumination of 1800–2000 lux (Letouvet-Pawlak et al. 1990). However, light of this intensity inhibited sporulation, but not fruiting body formation, of wild-type cells, rendering the results of this experiment inconclusive (data not shown). No other suitable regulated promoters have been identified for *M. xanthus*.

Ectopic expression of key regulatory genes sometimes induces development at inappropriate times. For example, ectopic expression of *brlA* induces conidiophore development of *Aspergillus nidulans* in the absence of nutritional stress (Adams et al. 1988). When cells containing the *carQRS-csgA* fusion (pBUL31) were placed on CTT agar, which contains 1% casitone to suppress development, neither aggregation nor sporulation was observed in the presence of light. These results suggest that ectopic *csgA* expression does not induce fruiting body morphogenesis in the absence of nutritional stress. A second way to test whether high levels of *csgA* expression induce aggregation in the absence of nutritional stress involves inducing *csgA* expression with certain peptidoglycan components that are known to stimulate rippling (Shimkets and Kaiser 1982). Wild-type cells containing the *csgA* reporter plasmid pLJS94 were grown in a thin film submerged under a layer of growth media containing the ripple inducers *N*-acetylglucosamine, *D*-alanine, and diaminopimelate (Fig. 4). The peptidoglycan components strongly stimulated *csgA* expression relative to unsupplemented controls, with the peak level of β -galactosidase specific activity at ~66% maximum *csgA* expression. This level of expression should have induced aggregation and substantial levels of sporulation according to the results obtained in Figures 2 and 3, but

Table 1. Effect of the *carQRS* promoter on sporulation and *csgA* expression

Construct	Structure ^a			β -Galactosidase (sp. act.) ^b	Sporulation (% LS523 with pLJS80) ^b
	artificial promoter	<i>csgA</i> regulatory region	structural gene		
pLJS80	none	–2400 to +1	<i>csgA</i>	N.A.	100
pLJS94	none	–2400 to +1	<i>lacZ</i>	38	N.A.
pLJS58	none	–400 to +1	<i>csgA</i>	N.A.	<1
pLI227	none	–400 to +1	<i>lacZ</i>	18	N.A.
pBUL31	<i>carQRS</i>	–400 to +1	<i>csgA</i>	N.A.	107
pDAH217	<i>carQRS</i>	none	<i>lacZ</i>	42	N.A.

^aPlasmids are listed as pairs with similar upstream regulatory regions but different structural genes. The *lacZ* gene was used to assess expression while the *csgA* gene was used to assess sporulation. pBUL31 was constructed by ligating the *carQRS* promoter to the *KpnI* site in the *csgA* upstream regulatory region. pDAH217 was kindly furnished by David Hodgson and contains the *lacZ* gene under the control of the *carQRS* promoter.

^b[N.A.] Not available.

only rippling was observed in these experiments. These results suggest that *csgA* is necessary but not sufficient for induction of aggregation and sporulation, which remain inhibited by the presence of excess nutrient. This possibility is also suggested by the fact that developing cells can resume vegetative growth when nutrients are added (data not shown). Therefore, it is likely that there are many points in the developmental pathway that can be suppressed with high nutrient, and at least some of these steps follow *csgA* expression in the developmental pathway.

Regulation of *csgA* expression

Because temporal regulation of *csgA* expression appears

Figure 4. Induction of *csgA* expression by peptidoglycan components. Wild-type strain DK1622 containing pLJS94 was induced to ripple in submerged culture with the peptidoglycan components *N*-acetylglucosamine, diaminopimelate, and *D*-alanine, as well as 0.2% casitone to repress fruiting body development. β -Galactosidase specific activity was measured at 24-hr intervals (■). (●) The time course of *csgA* expression during fruiting body development in submerged culture in the absence of exogenous casitone or peptidoglycan components. β -Galactosidase specific activity is given as nmoles of *o*-nitrophenol produced per minute per milligram of protein and is the average of three experiments with variations of <10%.

to be critical to fruiting body morphogenesis, environmental factors controlling development were tested to determine whether they regulate *csgA* expression. Development may be induced by limiting the carbon or phosphate source (Manoil and Kaiser 1980). Casitone serves as a rich source of carbon and nitrogen for *M. xanthus* and strongly inhibits *csgA* expression (data not shown). To further assess the role of nutrient limitation on *csgA* expression, cells containing each of the deletion constructs were placed on TPM agar, which is devoid of carbon and nitrogen, and compared with cells on CF agar, which is TPM agar plus low levels of NH_4^+ , pyruvate, and citrate. Wild-type cells containing pLJS94 produce a similar level of *csgA* expression and spore number on both types of agar (data not shown). However, sporulation proficiency and *csgA* expression of cells containing many of the deletion constructs were substantially different on the two types of agar (Fig. 5). The upstream region extending to –400 bp appears to be all that is necessary for optimal *csgA* expression and sporulation under conditions of extreme starvation on TPM agar, whereas a much larger region extending to –930 bp appears to be necessary to achieve the same results in the presence of low levels of nutrients on CF agar. Cells containing plasmids pLI226, pLJS63, and pLJS58, which form substantial numbers of spores on TPM agar, cease development during aggregation on CF agar and begin to disperse from the nascent fruiting bodies after ~96 hr.

It appears that the region extending from –400 to –930 bp can stimulate *csgA* expression in the presence of low levels of nutrients. Individual components were omitted from CF agar to determine which nutrients repress *csgA* expression of strains containing truncated regulatory regions. Elimination of either carbon source (pyruvate or citrate), the nitrogen source (NH_4^+), or the phosphate source (potassium phosphate) resulted in spore levels similar to those observed on TPM agar for plasmids pLI226, pLJS63, pLJS58, and pLJS91 (data not shown). These results suggest that the *csgA* upstream region is coupled to carbon-, nitrogen-, and phosphate-sensing systems.

Control of *csgA* expression by cell–cell interactions was assessed by transducing pLJS94 into a variety of oth-

Figure 5. Sporulation proficiency and *csgA* expression of cells containing the *csgA* deletions on CF and TPM agar. Sporulation was assayed using the constructs with the *csgA* structural gene; *csgA* expression was assayed using the constructs with the *lacZ* structural gene. The x-axis refers to the 5' end point of the cloned *csgA* regulatory region relative to the start site of transcription, which is designated +1. The restriction sites used in the cloning are given by the abbreviations along the x-axis: (N) *NarI*; (K), *KpnI*; (R), *EcoRI*; (A), *ApaI*. (■) *csgA* expression on CF agar; (●) *csgA* expression on TPM agar; (□) sporulation on CF agar; (○) sporulation on TPM agar. Values represent averages from three experiments, and the variation between experiments was < 10%.

erwise isogenic cell-signaling mutant strains and measuring β -galactosidase specific activity during the course of development. The *bsgA* mutation, which is involved in the production of the B signal (Gill and Bornemann 1988; Gill et al. 1988), reduced *csgA* expression at all stages of development, suggesting that *bsgA* affects both early and late phases of *csgA* expression (Fig. 6). *csgA* expression was reduced to a lesser extent in *csgA* cells, and only during sporulation. The *dsp*, *asgA*, and *dsgA* cells produced normal or slightly enhanced levels of *csgA* transcription (data not shown). These results suggest that the B signal and perhaps the C signal also contribute to the temporal control of *csgA* expression.

DNA sequence of the *csgA* upstream region

The nucleotide sequence of the entire *csgA* upstream region was determined on both DNA strands using the dideoxy chain-termination method. This sequence partially overlaps a portion of the *csgA* upstream DNA sequence reported previously by Hagen and Shimkets (1990) but is shown in its entirety to facilitate its analysis (Fig. 7). The *csgA* transcriptional start site is designated +1 at nucleotide 2400, and the likely translational start site is GTG beginning at nucleotide 2588. Computer analysis of this sequence was performed with the goal of identifying potential protein-coding regions in

the upstream sequence, as well as potential sites for binding of regulatory proteins.

Many *cis*-acting proteins are DNA-binding proteins that regulate expression of adjacent genes (for review, see McFall 1986). A putative protein-coding region with a codon usage typical of *M. xanthus* was observed in the opposite orientation of *csgA* initiated by the GTG codon at position 1903 and terminated with the TAG stop codon at position 905 (Fig. 7). This gene bears no sequence identity with other genes in GenBank. The location of this protein-coding region does not correspond with the boundaries of the *csgA* upstream region defined by the deletion analysis; it lies upstream of the DNA sequences necessary for maximal *csgA* expression on TPM agar while less than half of the protein is encoded in the region necessary for maximal expression on CF agar. Therefore, it does not appear as if the large upstream region encodes a protein that is required in *cis* for *csgA* expression.

The search for potential protein-binding sites in the *csgA* upstream region involved scanning the region for consensus sequences found in other gram-negative organisms. In enteric bacteria, the interaction of positively acting regulatory proteins with an upstream regulatory region is responsible for the transcriptional activation of families of nutritionally regulated genes. Some genes involved in carbon and energy utilization are regulated by the activated form of the CAP protein containing cAMP (for review, see Magasanik and Neidhardt 1987). No sequence with identity to the consensus CAP-binding domain, AANTGTGAN₂TN₄CA, was observed in the *csgA* upstream regulatory region. The most closely

Figure 6. Time courses of developmentally regulated *csgA* expression in *csgA*⁺ (■), *csgA*⁻ (□), and *bsgA*⁻ (○) cells. Plasmid pLJS94 was used to measure *csgA* expression on CF agar. β -Galactosidase specific activity units are given as nmoles of *o*-nitrophenol produced per minute per milligram of protein and represent the averages of two or three separate experiments. The values of each experiment are given with a horizontal slash, and the range is given with the vertical line.

Figure 7. Annotated nucleotide sequence of a *SalI*-*AvaII* fragment containing the *csgA* upstream regulatory region. Locations of 5' ends of the deletion plasmids are marked with the name of the plasmid above and the restriction sites below the nucleotide sequences. The *csgA* transcriptional initiation site T is marked as +1 at nucleotide 2400. The -35- and -10-bp regions of the potential promoter are overlined. The likely *csgA* initiator codon GTG is in bold print at nucleotide 2588. The underlined and bold CAC, at nucleotide 1903, and the bold CTA, at nucleotide 905, are the potential translational initiation and stop codons, respectively, of a gene transcribed in the opposite orientation; the underlined CTCC is the potential ribosome binding site for this protein, also in the complementary orientation. This sequence has been submitted to GenBank under accession number M73709.

matched sequence contained three mismatches and was located 613 nucleotides upstream from the *csgA* transcriptional start site at nucleotide 1787. It is unlikely that this sequence regulates *csgA* expression in a manner analogous to that of cAMP-CAP in enteric bacteria, as this sequence is not necessary for *csgA* expression under conditions of extreme carbon starvation on TPM agar.

In *E. coli*, nitrogen deficiency activates the expression of genes necessary to assimilate ammonium ions (for review, see Magasanik and Neidhardt 1987). The initiation of transcription from nitrogen-regulated promoters requires a novel σ -factor, σ^{60} , and an activator protein bound to the DNA at some distance from the promoter known as NRI or NtrC (Kustu et al. 1989; Reitzer and Magasanik 1986). The consensus promoter sequence of the nitrogen-regulated operons in enteric bacteria, TGGYAYRN₄TTGCA, is much different from that of the *csgA* promoter, suggesting that the *csgA* promoter is

not likely to be transcribed by the σ^{60} form of RNA polymerase. No NRI consensus-binding site, GCACN₅-TG GTGC, was identified in the *csgA* upstream regulatory region, although one sequence with two mismatches was observed beginning at nucleotide 1714. This sequence lies outside the region necessary for *csgA* expression under conditions of extreme nitrogen starvation. The upstream sequences of the *Klebsiella pneumoniae* *nif* promoters, which bind to the NifA activator protein instead of NRI, have a consensus sequence TGTN₄TN₅ACA (for review, see Magasanik and Neidhardt 1987). In the *csgA* upstream regulatory region, one sequence with a single mismatch was observed at nucleotide 1968, and two sequences with two mismatches were observed at nucleotides 2025 and 2338. Although these latter two sequences lie within the fragment required for maximal *csgA* expression under conditions of extreme nitrogen starvation, the absence of a σ^{60} -like

promoter makes it unlikely that they function in a manner analogous to the *nifA*-regulated promoters of *Klebsiella*.

Genes involved in phosphate assimilation have a common upstream regulatory region referred to as the phosphate box, CTG/TTCATAA/TAA/TCTGTCAC/T (for review, see Wanner 1987). No phosphate box-like sequences were observed in the *csgA* regulatory region, even with five mismatches. Therefore, the *csgA* upstream region appears to involve nutritional regulatory mechanisms substantially different from those of the enteric bacteria.

Discussion

The CsgA gene product was made limiting for development by constructing a series of nested deletions across the upstream regulatory region. Systematic reductions in the length of the *csgA* upstream regulatory region resulted in premature termination of development at earlier and earlier morphological stages as the result of decreased *csgA* expression. Conversely, artificial induction of *csgA* to levels observed during wild-type development resulted in quantitatively complete development. These results suggest that gradual increases in CsgA over the course of development entrain the morphological stages of development. Similar results were obtained with a different experimental approach in which purified CsgA was added to developing *csgA* cells (Kim and Kaiser 1991). Low concentrations of CsgA induced aggregation of *csgA* cells, whereas CsgA levels only 1.25-fold higher resulted in complete development. CsgA thus appears to be a morphogen, an inducing substance that can elicit different cellular responses at different concentrations (Slack 1987).

The presence of a large class of developmental mutants that sporulate but fail to aggregate argues that aggregation and sporulation are independent branches of the developmental pathway (Morrison and Zusman 1979). Because development ceases upon formation of the nonmotile myxospore, the overall success of fruiting body development depends on the timely induction of sporulation coinciding with the completion of aggregation. Because there are different thresholds for activation of each developmental stage by CsgA, gradual increases in *csgA* expression over the course of development appear to form the basis of a timing mechanism that entrains the morphological behaviors in their proper temporal order. CsgA production appears to be regulated at the level of transcription by a large upstream regulatory region that is coupled to an array of sensory systems that monitor nutrient concentration and cell-cell interactions. *csgA* transcription is inhibited by excess nutrients and stimulated by peptidoglycan components and the B signal. Modest levels of carbon, nitrogen, and phosphate do not interfere with *csgA* expression provided an upstream region extending 930 bp upstream from the transcriptional start site is present. The multiplicity of regulatory pathways, the large size of the regulatory region, and the small increases in *csgA* expression observed dur-

ing development suggest a complex regulatory mechanism involving multiple DNA-binding proteins.

There is tremendous diversity in the size and shape of the fruiting bodies within the myxobacteria (for review, see Shimkets 1990). Some species form fruiting bodies that are little more than mounds of spores. Fruiting bodies from other species contain spores on the top of a long stalk, whereas others resemble miniature trees in which the spores are contained in sporangioles at the end of branches. The evolution of such morphologically diverse structures could involve relatively few changes in the developmental program, particularly if they altered the timing of sporulation. By devoting more time to aggregation, fruiting bodies with more sophisticated structures could have time to form. Analysis of the regulatory regions of *csgA* alleles from other species might provide some clues concerning the evolution of fruiting body diversity.

Little is known about the mechanisms by which CsgA induces rippling, aggregation, and sporulation. Stimulation by CsgA presumably involves the activation of different temporal classes of developmental genes (Kroos and Kaiser 1987). One approach to the analysis of these regulatory circuits involved the isolation of sporulation-proficient *csgA* pseudorevertants, which fell into three phenotypic classes: those that restored development at the (1) rippling stage, (2) the aggregation stage, and (3) the sporulation stage (Rhie and Shimkets 1989). Analysis of these mutants and their regulatory regions may provide a more detailed understanding of the regulatory pathways involved with perception of the C signal.

Materials and methods

Bacteria and phage

M. xanthus cells were grown vegetatively at 32°C in CTT broth or on CTT agar (Hodgkin and Kaiser 1977) supplemented, when necessary, with kanamycin sulfate (40 µg/ml), or oxytetracycline (20 µg/ml). DK1622 (Shimkets and Kaiser 1982) is a *csgA*⁺ strain used as the wild-type. LS523 is a *csgA* null mutant generated by transposon insertion (Shimkets and Asher 1988). *E. coli* was grown in L broth or on L agar supplemented, when necessary, with kanamycin sulfate (50 µg/ml), ampicillin (50 µg/ml), or chloramphenicol (Cm) (12.5 µg/ml). The coliphage P1 *clr-100 cam* is a Cm^r, temperature-inducible variant of P1 (Rosner 1972) used to transduce plasmids from *E. coli* to *M. xanthus* by specialized transduction (Shimkets et al. 1983).

Induction and assay of development

For submerged culture development, exponentially growing cells were diluted in fresh CTT broth to 2 × 10⁷ cells/ml, and 8 ml was pipetted into a 100 × 15-mm plastic petri dish and incubated at 32°C for 20 hr. During this incubation the cells settled to the bottom, adhered, and grew to form a cohesive mat. The CTT broth was drawn off, and the cells were washed twice with an equal volume of distilled water. To induce rippling, the distilled water was replaced with 10 mM 3-*N*-morpholino-propanesulfonic acid (MOPS at pH 6.8), 1 mM CaCl₂, 0.2% (wt/vol) casitone (Difco), 1 mM of each of *N*-acetylglucosamine, diaminopimelate, and D-alanine. Rippling, fruiting body formation, and sporulation were also induced on agar plates. Vegetative

cells growing exponentially in CTT broth were resuspended in TPM buffer (10 mM Tris-HCl at pH 7.6, 8 mM MgSO₄, 1 mM potassium phosphate at pH 7.6) and spread uniformly at a density of 10⁷ cells/cm² on CF agar [10 mM Tris-HCl at pH 7.6, 1 mM potassium phosphate at pH 7.6, 8 mM MgSO₄, 0.2 mg of (NH₄)₂SO₄/ml, 150 µg of casitone/ml, 1 mg of Na-pyruvate/ml, 2 mg of Na-citrate/ml, and 1.5% Difco agar; Hagen et al. 1978] or TPM agar (TPM buffer plus 1.5% Difco agar).

For *carQRS* promoter induction with white light, cells were grown in CTT broth surrounded by a layer of aluminum foil to exclude extraneous light. Then, 5 × 10⁸ cells were spread on CTT or CF agar and positioned 12 cm from two 15 W fluorescent bulbs (General Electric, F15T8-SW). In dark control experiments, the plates were wrapped in aluminum foil and incubated under the fluorescent lights.

Aggregates and fruiting bodies were photographed after 48 hr of development on CF agar at 32°C using a Wild dissecting stereomicroscope. Because some strains make many small fruiting bodies and others make fewer large ones, the extent of aggregation was calculated from the total volume of the hemispherical fruiting bodies. The average volume of the fruiting bodies was computed from the measured diameter of >300 fruiting bodies. The total volume of the fruiting bodies is thus the average volume of the fruiting bodies times the number of fruiting bodies per plate.

Spore number was determined after 5 days of development, as described previously (Rhie and Shimkets 1989). Cells were scraped off the surface of the agar plates and subjected to sonic oscillation at 60 µW/cm² (Heat System Ultrasonics) for 10 sec to disrupt cell clumps. The myxospores were counted in a Petroff-Hausser counting chamber under a Leitz phase-contrast microscope.

β-Galactosidase assay

Changes in the β-galactosidase specific activity of a *csgA-lacZ* transcriptional fusion were used as an indirect assay for measuring changes in CsgA specific activity, which is much more difficult to quantify. The degree to which the reporter molecule concentration mimics that of the morphogen has yet to be assessed. CsgA is extracellular (Shimkets and Rafiee 1990) and appears to be relatively stable during development (Kim and Kaiser 1991). β-Galactosidase is cytoplasmic and seems to be unstable during development because the β-galactosidase specific activity decreases during the latter part of sporulation.

Cells were scraped off the surface of the submerged culture plates or agar plates with a razor blade, and the cell pellets were stored at -20°C until all of the time points were collected. Samples were thawed at room temperature, resuspended in buffer containing 0.1 M sodium phosphate (pH 7.6), 0.01 M KCl, 0.001 M MgSO₄, and 0.5 mM dithiothreitol, and sonicated on ice at 60 µW twice at 45-sec intervals with a microtip (Heat Systems Ultrasonics). Samples were centrifuged at 12,000g for 1 min to remove the cell debris, and the supernatant was assayed for β-galactosidase activity using the substrate *o*-nitrophenyl-β-D-galactoside as described by Kroos et al. (1986). Protein was assayed using the BCA reagents (Pierce Chemical Co.) with bovine serum albumin standards. β-Galactosidase specific activities are given as nanomoles of *o*-nitrophenol produced per minute per milligram of protein.

Nucleic acid manipulation

Plasmid DNA was manipulated by conventional techniques (Sambrook et al. 1989). Each of the *csgA* deletions was cloned into vector pLJS49 or its derivatives (Li and Shimkets 1988;

Shimkets and Asher 1988) in the region of the plasmid containing the *E. coli* phage P1 *parA* and *incA* sequences using the *Xho*I, *Hind*III, and *Eco*RI restriction sites. Previous studies have suggested that this region is transcriptionally inactive in *M. xanthus*, as a promoterless *lacZ* gene was not expressed in either orientation (pLJS64 in Li and Shimkets 1988; pLI225 in Fig. 2). Each plasmid was transduced into cells containing a *csgA* null mutation where it integrated into the bacteriophage Mx8 chromosome attachment site through site-specific recombination (Stellwag et al. 1985). The chromosomal location of the integrated plasmid was identified by Southern blotting (Southern 1975) or pulsed field gel electrophoresis (Chen et al. 1990, 1991).

Construction of plasmids pLJS51, pLJS58, and pLJS63 was described previously (Shimkets and Asher 1988). Plasmid pLJS80 was made by digesting pLJS43, a pUC19 derivative containing the *csgA* gene on a 5.4-kbp *Sal*I-*Sal*I fragment, with *Hind*III and ligating it into *Hind*III-digested pLJS49. Plasmids pLJS91, pLJS92, and pLJS93 were constructed from *Nar*I-partially digested *csgA* DNA, which was inserted into pUC19 and then inserted into the *Hind*III site of pLJS49. Plasmid pLJS94 and the other *lacZ* derivatives were constructed from pLJS45, which contains Tn5 *lac* inserted in the *csgA* protein-coding region (Shimkets and Asher 1988; Hagen and Shimket 1990). Because pLJS45 contains a truncated *csgA* upstream region, it was digested with *Apa*I and *Hind*III, and the 11.8-kbp fragment containing the *csgA-lacZ* fusion was ligated into *Apa*I-*Hind*III-digested pLJS43 to generate pLJS90, which was used to construct the entire *csgA-lacZ* series of clones.

Clones of specific DNA fragments were used as templates for DNA sequencing by the dideoxy chain-termination method (Sanger et al. 1977), with T7 DNA polymerase or *Taq* DNA polymerase.

The isolation of RNA was performed as described by Li and Shimkets (1988) from developing cells on TPM agar plates using a urea buffer [8.0 M urea, 350 mM NaCl, 50 mM Tris-HCl at pH 7.5, 20 mM Na₂ EDTA, 2% (wt/vol) Sarkosyl, 5% (vol/vol) phenol] and phenol extraction. The single-stranded synthetic oligonucleotide 5'-dGACCGGTACACACGTTGGTG3', complementary to a region extending from position 606 to 625 in the sequence of Hagen and Shimkets (1990) and 2308 to 2327 in this sequence (GenBank nucleic acid accession number M73709), was 5'-end-labeled with [γ-³²P]ATP using polynucleotide kinase. Oligonucleotide was added to 50 µg of total RNA in a volume of 10 µl containing 50 mM Tris (pH 8.0), 100 mM KCl, and the mixture was heated to 95°C for 3 min with subsequent annealing at 65°C for 2 min and cooling at room temperature for 5 min. The extension reactions were performed at 42°C for 60 min using avian myeloblastosis reverse transcriptase in 30 µl of total volume containing 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 50 mM Tris (pH 8.3), 40 mM KCl, and 60 mM MgCl₂. One microliter of 0.5 M EDTA and 1 µl of 1 mg/ml of pancreatic ribonuclease A were added to the reaction and digested at 37°C for 10 min. The reaction mixture was ethanol precipitated, resuspended in TE buffer, and fractionated on an 8% polyacrylamide sequencing gel in parallel with dideoxynucleotide sequencing reactions primed with the same oligonucleotide.

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