The *Myxococcus xanthus* Developmentally Expressed *asgB*-Dependent Genes Can Be Targets of the A Signal-Generating or A Signal-Responding Pathway

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Functional *Myxococcus xanthus* A signal-generating and A signal-responding pathways are required for the progression through early multicellular development. To identify genes responsive to these pathways, the expression of eight early developmental genes was analyzed. This examination identified one gene as a target of the A signal-generating pathway and four genes as targets of the A signal-responding pathway.

For a signaling pathway to be fully characterized, all its elements must be identified. The elements will include a signal, a receptor, transducers, and at least a subset of the responsive genes. In certain systems the organism itself generates the signal. An example of this is the *Myxococcus xanthus* A signaling system required for multicellular fruiting body development. In such cases of autogenous signal generation, two signaling pathways arranged in series must be considered. These are a signal-generating pathway and a signal-responding pathway. Thus, there will be two signals, two receptors, two sets of transducers, and two sets of responsive genes.

The signal-generating and signal-responding pathways are closely connected. The responding pathway is dependent on the presence of the signal. As a result, a defect in the signal-generating pathway will affect the responding pathway. Specifically, the loss of a positive regulator of signal generation will block the expression of *gpt* (for “generating pathway target”) genes and *rpt* (for “responding pathway target”) genes. Clearly, responsive genes in both the generating and responding pathways are dependent on the regulators of signal generation.

We have developed a scheme to differentiate *gpt* and *rpt* genes in relatively simple systems, such as *M. xanthus* A signaling, in which signal production is directly proportional to cell density. We hypothesized that *gpt* gene expression should be directly related to the generation of the signal. In strains defective in signal generation, the *gpt* genes should not be expressed and addition of the signal should not restore *gpt* expression. In contrast, *rpt* gene expression should be directly related to signal activity. In strains defective in signal generation, the *rpt* genes should not be expressed but addition of the signal should restore *rpt* gene expression.

We tested this hypothesis on the *M. xanthus* A signaling system by distinguishing possible *gpt* and *rpt* genes from a set of eight genes expressed during early development. The expression of these eight genes is greatly reduced in strains deficient in the production or activity of the *Asg* regulators of A signal generation (15). These genes are all termed *asg* dependent. This study classified one gene as a *gpt* gene and four genes as *rpt* genes.

Generation of and response to extracellular A signal are required for the progression through early *M. xanthus* devel-

opment (11). Extracellular A signal is a specific set of amino acids at an extracellular concentration greater than 10 μ M (16). The A signal functions to monitor cell density during starvation, permitting the cells to sense that a minimal cell density ($>3 \times 10^8$ cells per ml) has been reached and development can proceed (17). The A signal is generated, during the first hours of development (15, 16), when proteinases degrade the proteins on the surface of the cell in response to nutrient limitation (21). Each *M. xanthus* cell generates a fixed amount of A signal, so that the total amount is directly proportional to the number of cells per unit volume (9, 17). This is in contrast to certain other cell density-sensing systems, such as *Vibrio fischeri* autoinduction, in which the production of the signal is induced by a positive feedback loop (5). Three regulators required for A signal generation are encoded by the unlinked genes (14) *asgA* (22), *asgB* (20), and *asgC* (2). Strains containing mutations in any one of the genes generate A signal at less than 5% of the wild-type level (14) and release extracellular proteins at similarly low levels (21). These mutants are arrested very early in development (8). Certain aspects of development can be restored to *asg* mutants by the addition of extracellular A signal. This includes the restoration of gene expression and aggregation in all of the *asg* mutants and partial restoration of sporulation in *asgB* and *asgC* mutants (2, 17, 21). These data suggest that A signal is primarily responsible for the *asg*-dependent developmental arrest.

The *asg* genes are well characterized. The *asgA* gene encodes an unusual member of the two-component regulatory systems, in which two domains normally encoded by different genes are fused (22). The *asgB* gene encodes a putative DNA-binding protein that contains a helix-turn-helix region most similar to the -35 promoter interaction region of major sigma factors (20). The *asgC* gene encodes the *M. xanthus* major sigma factor, *rpoD* (2). Although the regulation may be complex, these genes positively regulate the expression of most developmentally expressed genes (15). The *asg* gene products are likely to respond directly to starvation by activating the expression of genes that generate the A signal (2, 20, 22). Potential targets include genes involved in proteinase synthesis and secretion (14). It is expected that a variety of target genes exist and that mutagenesis of most individual genes will not alter signal production significantly.

Previous to this report, the *asg*-dependent genes were all considered to be in the A signal-responding pathway. This was based on the ability of whole cells to rescue expression of a number of these genes (15). However, the ability of bona fide

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TABLE 1. Tn5 *lac* insertion strains of *M. xanthus*

Tn5 <i>lac</i> insertion ^a	<i>asg</i> ⁺ derivative ^b	<i>asgB480</i> derivative ^c	Expression time (h) ^d	Reference
Ω4273	DK4285	DK4281	4–6	15
Ω4411	DK4310	DK4312	2–3 (2–2.5)	15
Ω4427	DK4366	DK4371	6–8	15
Ω4442	DK4442	DK4379	1–1.5 (1–1.25)	15
Ω4457	DK4457	DK4376	3–4	15
Ω4494	DK4386	DK4389	2–3 (2.25–2.5)	15
Ω4521 ^e	DK4322	DK4324	1–1.5 (1–1.25)	15
Ω4530	DK4530	NA ^f	1–1.5 (1–1.25)	13

^a Each Tn5 *lac* fusion is inserted into a region of the *M. xanthus* chromosome referred to here for simplicity as a gene and designated by its Tn5 *lac* fusion insertion number.

^b The genotype of the *asg*⁺ background is *sglA1*.

^c The genotype of the *asgB480* background is *sglA1 asgB480*.

^d Expression time of each fusion in an *asg*⁺ background developing on starvation agar is shown. Expression time in starvation buffer is shown in parentheses if different from that on agar. The expression time is calculated as in the work of Kroos et al. (13). The linear portion of each curve was extrapolated down to a horizontal line drawn through the level of β-galactosidase activity present in growing cells. The point of the intersection of the lines approximates the time in which β-galactosidase expression begins to increase.

^e The Tn5 *lac* Ω4521 was also examined in DK6621, the *sglA1 asg480 sasA1* strain (10).

^f NA, not available.

A signal to rescue gene expression was confirmed only for the 4521 gene (16). As more information became available, particularly the observation that the *asg* genes encoded regulatory proteins (2, 20, 22), it appeared to be possible that some of the *asg*-dependent genes might be involved in the generation of A signal.

To determine if any of the earliest *asg*-dependent genes could be classified as *gpt* genes, the phenotypes of eight strains containing different *asg*-dependent Tn5 *lac* fusions were examined. Expression of each fusion begins at a characteristic time between 1 and 8 h after the initiation of development in wild-type cells (Table 1). These fusion-containing strains all form wild-type fruiting bodies (13). It is possible that the Tn5 *lac* insertions are downstream of the protein-coding regions, that the functions of these genes are redundant, or that the products of these genes are not essential for development. It was expected that *gpt* genes would be cell density independent, not rescued by extracellular A signal at low cell density, and expressed very early during development. It was expected that *rpt* genes would be cell density dependent, rescued by extracellular A signal at low cell density, and expressed after 1 h of development.

Cell density-dependent gene expression. One of the characteristics that should differentiate the *gpt* and *rpt* genes is their cell density dependence. Extracellular A signal is a cell density signal that accumulates in direct proportion to cell density (17). Thus, the *gpt* genes are expected to be expressed at a constant rate after the initiation of development. This expression should be independent of cell density. In contrast, the expression of *rpt* genes is expected to increase when extracellular A signal reaches its threshold concentration ($10 \mu\text{M}$) as the cell density exceeds 3×10^8 cells per ml (17). The only well-characterized *rpt* gene, 4521, exhibits this expression pattern. Its expression is low at densities below 3×10^8 cells per ml and then increases dramatically as the density is increased to 1×10^9 cells per ml (17) (Fig. 1A).

The cell density-dependent expression of each fusion was tested by the method of Kuspa et al. (17). Briefly, exponentially growing cells, containing one of the eight early *asg*-dependent Tn5 *lac* fusions in an *asg*⁺ background, were incubated at

FIG. 1. Cell density-dependent gene expression of *asg*-dependent genes. *M. xanthus asg*⁺ cells containing a Tn5 *lac* insertion in an *asg*-dependent gene were incubated at different cell densities in starvation buffer as described by Kuspa et al. (17). In panel C, the expression pattern of the 4521 gene in DK6621, an *asgB480 sasA1* background, is shown. The expression of each fusion was calculated from the increase in β-galactosidase activity per cell after 20 h of incubation. The results are presented as a percentage of the maximum activity each fusion reached. Each strain was tested in at least three independent experiments, and results of a representative experiment are shown.

different cell densities in starvation buffer. The β-galactosidase activity was measured after 20 h of incubation and normalized to cell number.

The results were striking and separated the genes into two groups (Fig. 1). Six genes, including 4521, showed cell density-dependent increased expression. Expression of five genes (4427, 4442, 4457, 4494, and 4521) was at a relatively low constant level up to cell densities of 5×10^8 /ml. Expression per cell then increased dramatically as the cell densities were increased to 1×10^9 /ml. The expression of one gene, 4411, was at an intermediate level up to a density of 5×10^8 /ml. Expression of this gene also increased as the density was increased to 1×10^9 /ml. Expression of four genes (4411, 4427, 4457, and 4521) decreased at densities greater than 1×10^9 cells per ml (Fig. 1A). Expression of two other genes (4442 and 4494)

decreased at densities greater than 3×10^9 cells per ml (data not shown). The decrease in expression at higher cell densities may be due to the accumulation of extracellular amino acids to levels above the starvation threshold. The presence of amino acids at concentrations greater than 1 to 10 mM limits 4521 expression (1, 16). The decrease in expression at two different cell densities suggests that these genes may have different sensitivities to the starvation signal or mechanisms for adaptation to extracellular A signal.

The expression of two genes (4273 and 4530) showed a different response to the increase in cell density (Fig. 1C). The expression of these genes decreased as the cell density was increased. Interestingly, the 4273 and 4530 expression patterns are strikingly similar to that of 4521 in a suppressor background, DK6621, in which 4521 expression is A signal independent (10) (Fig. 1C). These data suggest that the expression of 4273 and 4530 is A signal independent.

Phenotypic rescue of gene expression by the addition of A signal. A second characteristic that should differentiate *gpt* and *rpt* genes is the phenotypic rescue of gene expression at low cell density by added A signal. At low cell densities, in both *asg*⁺ and *asg* cells, extracellular A signal can not accumulate. Under these conditions, the expression of *gpt* genes should be relatively unaffected by the addition of A signal, whereas the expression of *rpt* genes should be restored.

To test the phenotypic rescue of gene expression by the addition of A signal, the method of Kuspa and Kaiser (14) was used. Cultures of cells containing one of the eight early *asg*-dependent Tn5 *lac* fusions in an *asg*⁺ or *asg* background were incubated at 2.5×10^8 cells per ml in starvation buffer. The buffer was supplemented with various concentrations of the A-signal amino acid proline or the supernatant from *asg*⁺ cells starved at high density. The β -galactosidase activity that had accumulated after 20 h of incubation was compared with the maximum level that fusion reached when the cell density was varied.

The addition of A signal phenotypically rescued the expression of a subset of genes (Fig. 2). Four cell density-dependent genes (4442, 4457, 4494, and 4521) were strongly rescued (70 to 300%) by A signal. Surprisingly, two of the cell density-dependent genes (4411 and 4427) were rescued poorly by A signal (a maximum of 30%). The two genes (4273 and 4530) whose expression patterns at various cell densities appeared to be A signal independent were, as expected, poorly rescued by A signal (a maximum of 50%). In most cases, the response to the addition of A signal was similar whether the fusions were in an *asg*⁺ or *asg* background. Generally, the phenotypic rescue of gene expression by high levels of proline and that by the wild-type developmental supernatant were similar.

Developmental expression times. A-signal activity increases dramatically at about 1 h after the initiation of development (15, 16). Thus, any *gpt* genes would be expected to increase expression during the first hours of development. In contrast, *rpt* genes would be expected to increase expression after 1 h of development. The expression time of each fusion in an *asg*⁺ background was estimated by measuring at intervals of 15 min, 30 min, or 1 h the β -galactosidase specific activity in cells developing in starvation buffer and on starvation agar plates. The expression of each gene (Table 1) was similar under the two conditions. The expression times of the two genes (4273 and 4530) whose cell density-dependent expression patterns appeared to be A signal independent were strikingly different from each other. The 4530 gene had an expression time of 1 to 1.5 h, and the 4273 gene had an expression time of 4 to 6 h. These results suggest that 4530 may be a *gpt* gene, but this is unlikely for 4273. Interestingly, 4273 is the only gene of this

FIG. 2. Phenotypic rescue of gene expression by the addition of A signal to *asg*⁺ and *asgB* cells at low cell density. Cultures of *asg*⁺ and *asgB480* cells each containing a Tn5 *lac* insertion in a different *asg*-dependent gene were incubated at 2.5×10^8 /ml in starvation buffer supplemented with increasing concentrations (0.1, 0.25, 0.5, and 1 mM) of the A-signal amino acid proline or the supernatant (0.4 ml) from *asg*⁺ cells developing at high density (4×10^9 /ml) as described by Kuspa and Kaiser (16). β -Galactosidase activity was measured at 20 h. The results are presented as a percentage of the maximum β -galactosidase activity each fusion reached as the cell density was varied. Black bars represent *asg*⁺ strains and white bars represent *asgB480* strains. Each bar represents an average of three experiments. The range is shown by the error bars.

group of eight in which a Tn5 *lac* fusion was generated by insertion into a previously identified gene (13). This gene is a spore coat protein gene, *tps* (4). The regulation of *tps* expression by the *asg* genes is likely to be unrelated to A signaling. The expression times of the four *rpt* genes are between 1 and 4 h. The genes 4411 and 4427, which are cell density dependent and showed poor phenotypic rescue at low cell density by the addition of A signal, have expression times of 2 to 3 h and 6 to 8 h, respectively.

One A signal-generating-pathway target gene and four A signal-responding-pathway target genes are distinguished. These analyses separate the *asg*-dependent genes into four classes (Fig. 3). One gene, 4530, is a likely target for the A signal-generating pathway. It is surprising that only one putative *gpt* gene was found. Perhaps the majority of the *gpt* genes are expressed during growth and development; genes of this

FIG. 3. The *asg*-dependent genes include genes responsive to the A signal-generating and A signal-responding pathways. An individual *M. xanthus* cell is represented during development to illustrate the predicted placement within the A signaling system of eight *asg*-dependent genes expressed during early development. Arrows indicate positive biochemical or genetic functions. Nutrient limitation is sensed, at least in part, by a rise in the levels of intracellular guanosine penta- and tetraphosphate, represented by (p)ppGpp (23). Boxed genes are required for A signal generation (2, 14, 20, 22). The 4530 gene appears to be responsive to the A signal-generating pathway and is likely to be a target of the *asg* genes. The 4273 gene appears to be regulated by the *asg* genes independently of A signal. The 4442, 4457, 4494 and 4521 genes are responsive to the A signal-responding pathway. The 4427 and 4411 cell density-dependent genes appear to be regulated by mechanisms other than A signaling.

type were not examined. Interestingly, 4530 is unusual, being the only gene among 12 developmentally expressed Tn5 *lac* fusions tested to be induced by the addition of glycerol (0.5 M) to the growth medium (3). These conditions result in fruiting body-independent production of a characteristic type of spore. These data suggest that either 4530 expression is controlled by two pathways or the *asg* genes mediate glycerol-induced sporulation. The former possibility is the most likely because the *asg* mutants are not significantly altered in their ability to form glycerol-induced spores (1, 19). The *asg* dependence of *tps* that appears to be A signal independent is also intriguing. It supports the idea that the *asg* genes control more than just extracellular A signal production.

The 4521 gene and three other genes (4442, 4457, and 4494) are *rpt* genes. Perhaps some of these genes are primary targets of the A signal transduction pathway. The 4521 gene, which is among the first of these genes to be expressed during development, is a likely candidate. Previously, 4521 was the best characterized *asg*-dependent gene and it was used to monitor the A signaling system. A number of the regulators of 4521 have been identified (7, 10), and its transcript start site (12) and minimum upstream regulatory region (6) have been determined. The 4521 promoter is a member of the family of sigma 54-dependent promoters (12). This suggests that for open complex formation an activator will bind to the upstream regulatory region (18). It will be interesting to examine the regulation of the other *rpt* genes (4442, 4457, and 4494) and to compare their regulation with that of 4521.

The regulation of genes 4411 and 4427, which are cell density dependent and appear to be A signal independent, should also prove interesting. These genes can be considered A signal independent because they show poor phenotypic rescue at low cell density by the addition of A signal, whether it is supplied by the A-signal amino acid proline or by extracellular supernatants of *asg*⁺ cells (Fig. 2). Interestingly, 4411 expression can be rescued by the presence of whole wild-type cells (1, 15). In contrast, 4427 was not rescued under these conditions (1). It is

possible that 4411 expression is controlled by a cell-cell contact-mediated signal, whereas 4427 expression may respond to a physiological parameter such as the decrease in oxygen tension that accompanies a higher cell density. Bioassays using wild-type and *asg* mutant strains containing 4411 or 4427 Tn5 *lac* fusions as the tester cells should be useful in characterizing these responses.

We thank D. Kaiser and Y. Cheng for providing the strains used in this study. We also thank J. Pollard and C. Crook for technical assistance and A. Kuspa for comments on the manuscript.

This investigation was supported by Public Health Service grant GM47444 to H.B.K. from the National Institutes of Health.

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