# The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *Myxococcus xanthus*

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**Guanosine 3′-di-5′-(tri)di-phosphate nucleotides [(p)ppGpp], synthesized in response to amino acid limitation, induce early gene expression leading to multicellular fruiting body formation in *Myxococcus xanthus*. A mutant (DK527) that fails to accumulate (p)ppGpp in response to starvation was found to be blocked in development prior to aggregation. By use of a series of developmentally regulated Tn*5lac* transcriptional fusion reporters, the time of developmental arrest in DK527 was narrowed to within the few hours of development, the period of starvation recognition. The mutant is also defective in the production of A-factor, an early extracellular cell-density signal. The *relA* gene from *Escherichia coli*, which encodes a ribosome-dependent (p)ppGpp synthetase, rescues this mutant. We also demonstrate that inactivation of the *M. xanthus relA* homolog blocks development and the accumulation of (p)ppGpp. Moreover, the wild-type allele of *Myxococcus relA* rescues DK527. These observations support a model in which accumulation of (p)ppGpp, in response to starvation, initiates the program of fruiting body development, including the production of A-factor.**

[*Key Words:* Guanosine tetraphosphate; *Myxococcus xanthus* development; RelA; stringent response; A-factor]

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When Myxobacteria are deprived of essential nutrients, they undergo a developmental program in which ~100,000 cells aggregate to form a multicellular fruiting body. Individual cells within the fruiting bodies differentiate into environmentally resistant and metabolically quiescent myxospores. Previous work has demonstrated that carbon, nitrogen, or phosphate deprivation (Dworkin 1962; Wireman et al. 1977; Manoil and Kaiser 1980a; Manoil 1982; Shimkets 1984), but not purine or pyrimidine starvation (Kimsey and Kaiser 1991; Singer and Kaiser 1995) initiates fruiting body development. *M. xanthus* is bacteriolytic and feeds on the proteins, peptides, and amino acids of prey bacteria but is unable to utilize the sugars liberated because of the absence of certain glycolytic enzymes such as pyruvate kinase (Watson and Dworkin 1968). Studies probing the nutritional requirements of *M. xanthus* have demonstrated this organism's preference for amino acids and small molecular weight carbon compounds such as pyruvate, acetate (Bretscher and Kaiser 1978), and to a lesser extent certain Kreb's cycle intermediates (Watson and Dworkin 1968).

*M. xanthus* is unable to synthesize leucine, isoleucine, or valine. Starvation for any of these three essential amino acids, or of an auxotroph for its newly essential amino acid, or addition of a tRNA charging inhibitor (such as serine hydroxamate or tyrosinol) elicits a developmental response (Manoil and Kaiser 1980a,b). All of these conditions lead to a decrease in the corresponding charged tRNAs, suggesting a connection between the initiation of fruiting body development and a stringent response. The stringent response couples the availability of aminoacylated (charged) tRNA molecules to the rate of protein synthesis through the signaling molecules guanosine-5′-(tri)di-3′-diphosphate [(p)ppGpp] (for review, see Cashel et al. 1996). In *Escherichia coli*, for example, ribosomal and tRNA synthesis is immediately inhibited in response to amino acid limitation (Cashel 1969; Cashel and Gallant 1969; Stent and Brenner 1961). Accumulation of (p)ppGpp arrests synthesis of stable RNA, activates amino acid biosynthetic operons, increases proteolysis, and inhibits the synthesis of DNA, membranes, and cell walls (Cashel et al. 1996). In addition, recent work by Chakraburtty and Bibb (1997) has implicated (p)ppGpp as a positive regulator of antibiotic, pigment, and aerial mycelium formation in *Streptomyces coelicolor* A3(2).

Two independent lines of evidence support the hypothesis that (p)ppGpp is a general starvation signal for *M. xanthus*. First, Manoil and Kaiser (1980a,b) found that

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(p)ppGpp accumulates rapidly after transfer of growing cells to nutrient-limited conditions. Furthermore, all known conditions that initiate fruiting body development have been found to elicit an increase in intracellular (p)ppGpp concentration (Manoil and Kaiser 1980a,b). Second, we recently demonstrated that expression of the *E. coli relA*$^+$ gene in *M. xanthus*, even in the presence of ample nutrients, resulted in a simultaneous increase in (p)ppGpp accumulation and activation of early developmentally specific gene expression (Singer and Kaiser 1995). The finding that ectopic expression of *E. coli relA* and subsequent (p)ppGpp accumulation induces development implies that this nucleotide is sufficient to initiate the developmental program.

Before this work, the question remained whether (p)ppGpp is also necessary for development. We have taken two approaches to address this question. First, we have further examined the developmental block associated with a known mutant, DK527, which has lost the ability to accumulate (p)ppGpp in response to starvation (Manoil and Kaiser 1980a,b). Second, we initiated a search for the *M. xanthus* homolog of *relA*, which is responsible in *E. coli* and *Salmonella typhimurium* for ribosome-dependent (p)ppGpp synthetase activity, and have characterized the phenotype of a disruption in this gene with respect to development.

## Results

### *Mutant DK527, which fails to develop, uncouples RNA synthesis from amino acid availability*

DK527 was derived from DK101 (Table 1), which is capable of responding to starvation by accumulating (p)ppGpp, and subsequently forming fruiting bodies with spores (Manoil and Kaiser 1980b). The failure of DK527 to accumulate (p)ppGpp after starvation parallels a similar failure in the *relA*$^-$ (relaxed) mutants of *E. coli* (Borek et al. 1956; Stent and Brenner 1961; Fiil and Friesen 1968; Cashel and Gallant 1969). If DK527 is the *Myxococcus* version of a relaxed mutant, its stable RNA synthesis should be uncoupled from amino acid availability. To test this deduction, the synthesis of RNA by *M. xanthus* DK101 and the DK527 mutant strain were compared with that of wild-type *E. coli* and an *E. coli relA*$^-$ mutant. As shown in Figure 1A, when wild-type *M. xanthus* cells are abruptly deprived of seryl-tRNA by the addition of serine hydroxamate, a competitive inhibitor of seryl-tRNA charging in *E. coli* (Tosa and Pizer 1971), within 30 min total RNA synthesis decreases to ~50% of the pretreatment rate. Apparently, wild-type *M. xanthus*, like wild-type *E. coli* (Fig. 1B), interrupts stable RNA synthesis when seryl-tRNA is no longer available for protein synthesis. The response of *M. xanthus* is more gradual than that of *E. coli*, consistent with the ~10-fold lower rate of cell growth and RNA synthesis in *M. xanthus* as compared with *E. coli*. Serine hydroxamate induces an increase in the intracellular level of (p)ppGpp in *M. xanthus* (Manoil and Kaiser 1980a; Singer and Kaiser 1995). Thus, it appears that wild-type *M. xanthus*, like *E. coli*, couples stable RNA synthesis to aminoacyl tRNA availability. In contrast, when the DK527 mutant was exposed to serine hydroxamate, total RNA synthesis continued at the pretreatment rate (Fig. 1A), like the *E. coli relA* mutant (Fig. 1B).

### *The* E. coli relA *gene rescues the developmental defect of DK527*

The failure of DK527 to develop fruiting bodies in response to starvation accompanies its failure to accumulate (p)ppGpp. To determine whether the failure to develop results from the inability to accumulate (p)ppGpp, the *E. coli relA* gene was tested for its ability to restore development in DK527. *M. xanthus* DK101 and DK527 strains were transformed with plasmid pMS132, which carries the *E. coli relA* gene under the transcriptional control of the light-inducible *M. xanthus carQRS* promoter (Singer and Kaiser 1995). To provide negative controls, these same strains were transformed with plasmid pMS131, which is identical to pMS132 but lacks the *E. coli relA* gene (Singer and Kaiser 1995). Both plasmids (pMS131 and pMS132) have the myxophage Mx8 attachment site Mx8*attP*, which provides high-efficiency, site-specific integration at the chromosomal Mx8 *attB* site (Orndorff et al. 1984), and the *nptII* gene encoding kanamycin resistance. Following transformation, the presence of a single integrated copy of pMS131 or pMS132 in the *M. xanthus* genome was confirmed by Southern blot (data not shown). Inducible expression of *E. coli* RelA protein was demonstrated by Western blotting cultures with or without exposure to light (Fig. 2).

Plasmid-carrying derivatives of DK101 and DK527 were also examined for the initiation of fruiting body development when bacteria were starved and expression of *E. coli* RelA was induced by exposure to light. The *M. xanthus* DK527 strain containing the *E. coli relA*$^+$ gene regained the ability to form mounds when placed on starvation medium in the presence of light; plasmid-containing DK527 cells lacking *E. coli relA*$^+$ were, like DK527 itself, unable to form mounds. DK101 strains, with or without the *E. coli relA*$^+$ gene, were unaffected with respect to mound formation. It should be mentioned that under these conditions, development is inhibited at the mound stage (Li et al. 1992; Singer and Kaiser 1995).

Western Blot analysis (Fig. 2) shows that cultures before exposure to light (0 hr) were expressing a small amount of *E. coli* RelA protein; this lower level of *E. coli* RelA protein was sufficient to rescue fruiting body development of DK527 under starvation conditions. Cultures carrying the *E. coli relA*$^+$ gene were starved in the dark; they too had regained the ability to form fruiting bodies and to sporulate. DK527 strains carrying the control plasmid pMS131 (no *E. coli relA*$^+$) remained unable to form fruiting bodies. In addition to regaining the ability to develop in response to starvation, DK527 strains carrying *E. coli relA*$^+$ regain the ability to accumulate (p)ppGpp in response to starvation. This experiment implies that a relatively small amount of *E. coli* RelA pro-

**Table 1.** *Strains and plasmids*

| Plasmid | Construction or relevant markers | Source |
|---|---|---|
| G4 | *M. xanthus* cosmid | R. Gill (University of Colorado, Health Sciences, Denver) |
| D10 | *M. xanthus* cosmid | R. Gill |
| pBGS18 | cloning vector carrying $Km^r$ | Spratt et al. (1986) |
| pBluescript SK+ | cloning vector carrying $Ap^r$ | Stratagene |
| pTTQ18 | $P_{tac}$ expression vector | Amersham |
| pPLH323 | cloning vector carrying $Km^r$ and *Mx8attP* site | P Hartzell (University of Idaho, Moscow) |
| pSWU29 | cloning vector carrying $Tc^r$ and *Mx8attP* site | S. Wu (Stanford University, CA) |
| pPCR-A | 311-bp PCR fragment cloned into pBGS18 | this study |
| pMS131 | control for pMS131, lacks *E. coli relA* | Singer and Kaiser (1995) |
| pMS132 | *E. coli relA* gene under $P_{car}$ control | Singer and Kaiser (1995) |
| pMS300 | 1.2-kb *Sac*I fragment from pMS302 in pBGS18 | this study |
| pMS302 | 4.8-kb *Pst*I fragment from G4 in pBGS18 | this study |
| pMS320 | 4-kb *Pst*I–*Nco*I of pMS302 | this study |
| pMS321 | 4-kb *Pst*I–*Hin*dIII fragment from pMS320 cloned into pSWU29 ($Tc^r$) | this study |
| pMS323 | 1-kb *Sac*I–*Nco*I fragment from pMS320 into pBGS18 | this study |
| pMS325 | 480-bp *Eag*I (filled in)–*Xho*I fragment cloned into *Hin*dIII (filled in)–*Xho*I sites of pMS321 ($Tc^r$) | this study |
| pMS350 | 4.8-kb *Pst*I fragment from G4 into pPLH343 | this study |
| pMS379 | 3.2-kb *Xho*I–*Pst*I fragment into pBGS18 | this study |
| pMS380 | 267-bp *Bam*HI–*Xho*I PCR fragment cloned into pMS379 | this study |
| pMS381 | 3.4-kb *Bam*HI–*Pst*I fragment from pMS380 cloned into pTTQ18–RelA under $P_{tac}$ control. | this study |
| **Strains** | **Relevant genotype** | |
| *M. xanthus* | | |
| DK101 | *sglA1* | D. Kaiser (Stanford University, CA) |
| DK527 | DK101 *relA527* | Manoil and Kaiser (1980a) |
| DK10528 | DK527 pMS132 | this study |
| DK10529 | DK101 pMS132 | this study |
| DK480 | DK101*asgB480* | Kuspa et al. (1986) |
| DK4324 | DK101*asgB480* Ω4521 | Kuspa et al. (1986) |
| DK4521 | Ω4521 | Kroos et al. (1986) |
| DK4400 | Ω4400 | Kroos et al. (1986) |
| DK4408 | Ω4408 | Kroos et al. (1986) |
| DK4455 | Ω4455 | Kroos et al. (1986) |
| MS8 | DK101 *relA*::pMS302 (tandom duplication of *relA* region) | this study |
| MS9 | DK527*relA*::pMS302 (tandom duplication of *relA* region) | this study |
| MS10 | DK101 *relA*::*pMS300* | this study |
| MS11 | DK101 pMS321 | this study |
| MS12 | MS10 pMS321 | this study |
| MS13 | DK527 pMS321 | this study |
| MS15 | DK527 pMS325 | this study |
| MS16 | DK101 pMS325 | this study |
| *E. coli* | | |
| MG1655 | wild type | B. Bachman (Yale University, New Haven, CT) |
| CF1651 | *relAΔ251*::*kan* | M. Cashel (National Institutes of Health, Bethesda, MD) |
| MS-Ec12 | MG1655 pMS381 | this study |
| MS-Ec51 | CF1651 pMS381 | this study |

tein is sufficient to produce the (p)ppGpp needed to rescue DK527.

The DK527 mutant grows slowly, even in complete medium. Long term cultures accumulate strains—presumably with suppressor mutations—that can grow faster than DK527. Therefore, it was necessary to test whether the recovery of development in the strains that carried the *E. coli* $relA^+$ gene might be the result of a

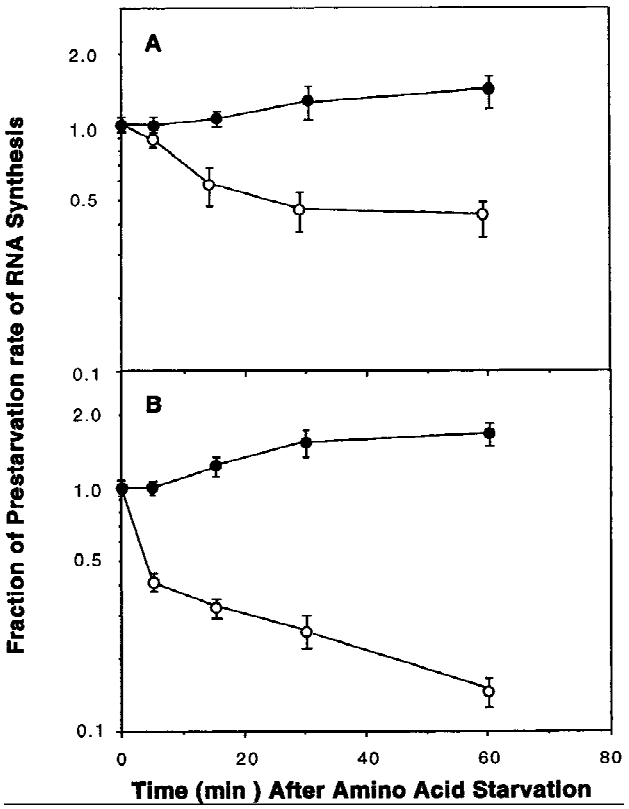


**Figure 1.** Effects of serine hydroxamate-induced serine starvation on RNA synthesis in *M. xanthus*, DK101 (○) or DK527 (●) (*A*), and *E. coli*, wild-type (○) or *relA*$^-$ (●) (*B*). Strains were grown at 30°C in either M9–glucose (*E. coli*) to an $OD_{450}$ of 0.2 to 0.3 or in M1 medium (*M. xanthus*) at 33°C to a density of 60–100 Klett units, as described in Materials and Methods. Samples were pulse-labeled at the indicated times (in min) with [$^3$H]uridine, and RNA synthesis rates were determined as described in Materials and Methods. Each time point records an average of three independent experiments.

second-site suppressor. To check this possibility, the plasmid pMS132 in strain DK10528 (DK527 pMS132) was replaced with pMS133, which lacks the *E. coli relA*$^+$ gene but encodes tetracycline resistance (Tc$^r$) (Singer and Kaiser 1995). Replacement was achieved by transduction and homologous recombination. Ten Tc$^r$ transductants of DK10528 were then screened with an *E. coli relA*$^+$ gene-specific probe by Southern blot (data not shown). All ten transductants had lost the *E. coli relA* gene. Seven of the ten transductants had lost the ability of DK10528 to develop, returning to the phenotype of DK527. Because seven of the ten transductants of DK10528 lost the ability to develop following exchange of pMS133 for pMS132, it is clear that the developmental competence of DK10528 was associated with the *relA*$^+$ gene and not with a second-site suppressor elsewhere in the genome. The three Tc$^r$ transductants that retained the Agg$^+$ Fb$^+$ phenotype may be caused by suppressor(s) that had arisen while those transductants grew on their Tc selection plate. These experiments confirm that rescue of the DK527 mutation by the *E. coli relA*$^+$ gene is the result of complementation by the gene and its protein product.

### *DK527 fails to express early developmental markers*

DK527 cannot undergo aggregation (Manoil and Kaiser 1980b), but wild-type cells (DK101) show their first morphological signs of aggregation at ~6 hr postinitiation. To define more precisely the stage at which the mutation in DK527 causes it to deviate from the normal developmental program, four well-characterized Tn*5lac* transcriptional fusion reporters, Ω4408, Ω4455, Ω4521, and Ω4400, representative of the early stages of development were examined in the DK527 genetic background. These reporters begin their β-galactosidase expression at different fusion-specific times in the 0–6 hr interval. No increase in β-galactosidase expression from any of these four reporter fusions was observed in DK527, as compared with DK101 (Fig. 3). Thus, the mutation in DK527 prevents cells from initiating early gene expression as defined by these Tn*5lac* reporters. These experiments confine the temporal block to within the first few hours of the normal developmental program. Because (p)ppGpp is synthesized then, this is expected to be the time when starvation is recognized and the decision to develop fruiting bodies is made.

### *Cloning the* M. xanthus *RelA homolog*

On the basis of the phenotype of DK527 and its complementation by the *E. coli relA* gene, a search for the *M. xanthus relA* homolog was initiated by use of a PCR-based approach. Taking advantage of regions of amino acid identity between the known amino acid sequences of RelA proteins from several different Eubacteria (Metzger et al. 1988; Sarubbi et al. 1989; Chakraburtty et al. 1996), PCR primers were designed to amplify DNA sequences encoding the *M. xanthus relA* homolog. A single 311-bp PCR product was detected by use of primers R-1750 and X-1125 (see Materials and Methods). DNA sequence analysis of this fragment predicted a protein coding sequence that was 49% identical and 75% similar to both the *E. coli* RelA and SpoT proteins, which are very similar to each other (Chakraburtty et al 1996).

The 311-bp PCR-generated fragment was used as a

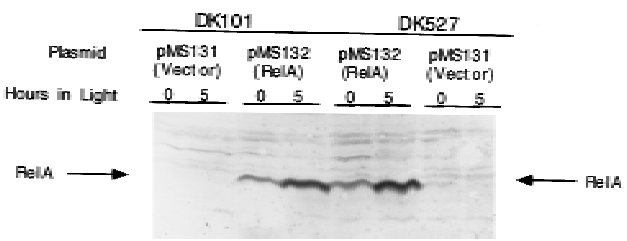


**Figure 2.** Expression of *E. coli* RelA in *M. xanthus* strains DK101 and DK527 under starvation in the dark (time 0) and after 5 hr of exposure to light. Western blotting is described in Materials and Methods. Arrows indicate the expected mobility of *E. coli* RelA protein.

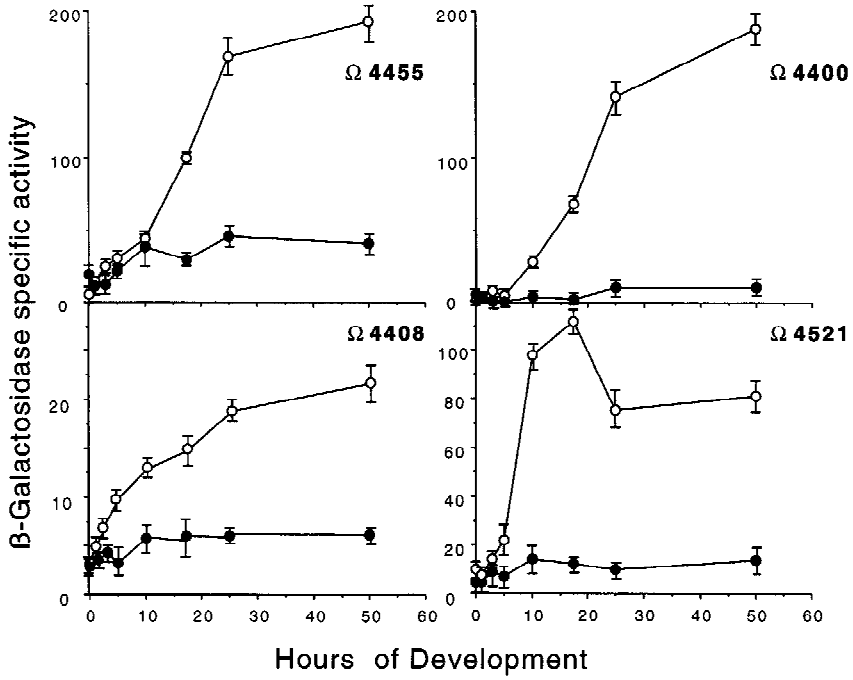


**Figure 3.** β-Galactosidase expression from Tn*5lac* fusions 4455, 4400, 4408, and 4521 after starvation in strains DK101 (○) and DK527 (●). Measurement of β-galactosidase specific activity is described in Materials and Methods; each point is an average of at least three independent experiments.

probe to screen an *M. xanthus* DNA cosmid library (generously provided by R. Gill, University of Colorado Health Sciences, Denver). This screen identified two cosmid clones, G4 and D10. Subsequent restriction endonuclease mapping, and Southern blotting of these cosmids revealed that they contained overlapping DNA and that the *M. xanthus relA* homolog resided on a common 4.8-kb *Pst*I fragment (data not shown). This 4.8-kb *Pst*I fragment was cloned into pBGS18; a restriction map of this region is diagrammed in Figure 4A. The sequence of the fragment has been submitted to Genbank, accession no. AF025847.

The 4.8-kb *Pst*I fragment is predicted to encode three ORFs, designated ORF 1–3 (Fig. 4A). ORF 2 exhibits

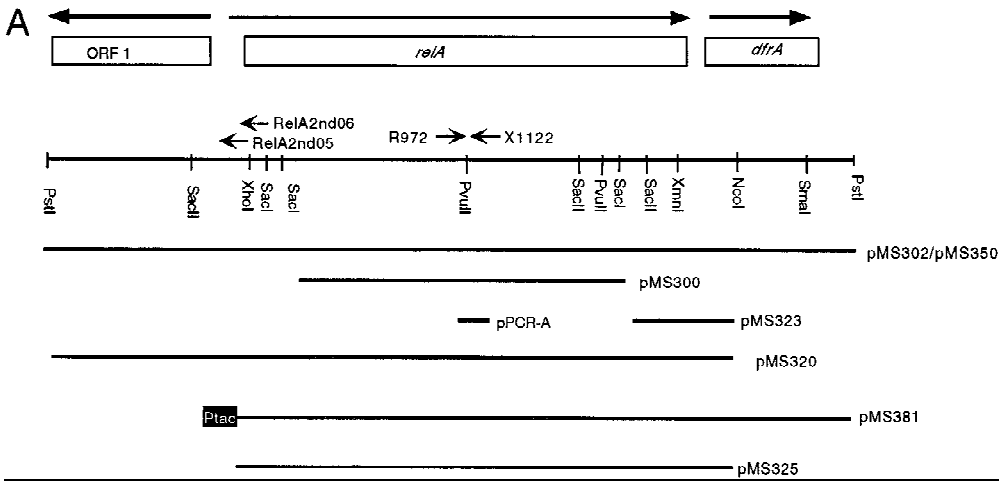


**Figure 4.** Analysis of the *M. xanthus relA* locus. (*A*) Restriction map of the *M. xanthus relA* region. Arrows above the boxes represent orientation of each gene and putative ORF. DNA sequences contained in plasmids used in this work are designated by lines below the restriction map followed by the plasmid designation (see Materials and Methods for construction details). The black box in pMS381 designates the $P_{tac}$ promoter region used to drive the *relA* gene. Forked arrowheads represent primers used for primer extensions and for PCR construction of pPCR-A. (*B*) (*See facing page.*) Sequence comparison between *M. xanthus* RelA and six other species. Alignments were performed by use of Macaw v. 2.0.5 from the National Center for Biotechnology Information, National Library of Medicine. Black boxes represent identical matches in all seven proteins; shaded boxes represent either five of seven matches or similar amino acid substitutions. (*M.x.*) *M. xanthus;* (*E.c.*) *E. coli;* (*V.s.*) *Vibrio* sp.; (*H.i.*) *H. influenzae;* (*Sn.*) *Synechocystis* sp.; (*S.c.*) *S. coelicolor;* (*S.e.*) *S. equi.*

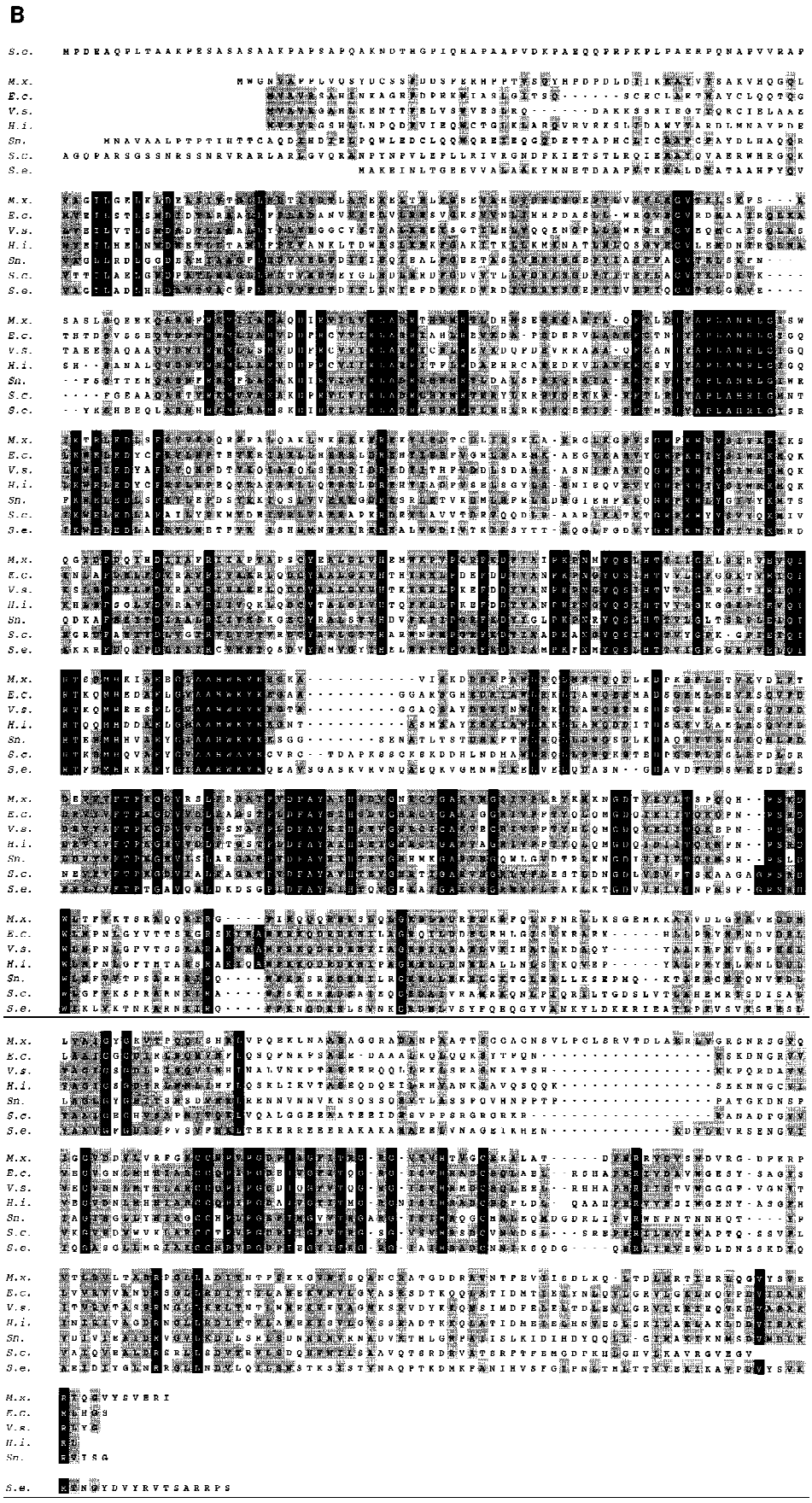


**Figure 4.** (*Continued; See facing page for legend.*)

strong similarity to RelA and SpoT; Figure 4B shows sequence similarity between ORF 2 and several bacterial RelA proteins. ORF 3 shows similarity to a group of small acid-soluble proteins implicated in translation inhibition, the YER057c/YJGF family (Oka et al. 1995; Ceciliani et al. 1996; Schmiedeknecht et al. 1996). Both putative ORFs would be transcribed in the same direction. Sequence analysis of DNA 5′ to the putative *relA/spoT* gene (which includes the putative ORF 1) shows no significant similarity to anything in the database when translated in all six potential reading frames.

Primer extension analysis was used to identify the transcriptional start site for the *relA/spoT* gene; a single 5′ end was identified (Fig. 5A). On the basis of the results of these experiments, on sequence similarity, and on the availability of a promising Shine–Dalgarno site nearby, we have placed the putative translational start at a GTG located 106 bp downstream from the transcriptional start site (Fig. 5A); this *relA* gene would encode a protein of 757 amino acids with a formal molecular mass of 85 kD. Sequence analysis also revealed a potential 8-bp DNA stem and 3-bp loop structure located between *relA* and *dfrA* ($\Delta G = -18.3$ kcal), followed by a run of three T residues (Fig. 5B). This putative stem-loop structure is located 15 bp from the presumed translational start of *dfrA* and could represent a transcriptional terminator for the *relA* gene.

### *Locating the* relA *gene on the* M. xanthus *physical map*

By use of the 311-bp PCR-generated fragment described above as a probe for Southern blot analysis of *M. xanthus* chromosomal DNA, two unique restriction fragments were identified. This implies that *M. xanthus,* like *E. coli,* may have one *relA* gene and one *spoT* gene. In *E. coli,* the *relA* and *spoT* genes are physically separated and map at 60 and 82 min, respectively. To determine whether the two putative *M. xanthus* genes were physically separated, their locations were mapped to the physical *M. xanthus* genome map (Shimkets 1993). The PCR-generated fragment was used as a probe to determine the position of each putative gene by use of pulsed-field electrophoresis (Chen et al. 1990). This analysis placed one of the genes at position 1–1.3Mb (*Ase*I fragment N and *Spe*I fragment U′) and the other at position 4.8–5Mb (*Ase*I fragment P and *Spe*I fragment S) on the 9.5-Mb *M. xanthus* physical map (Fig. 6). To determine the precise location of the cloned gene, the entire 4.8-kb *Pst*I fragment was used to probe DNA isolated from an ordered *M. xanthus* YAC library (Kuspa et al. 1989). Hybridization results indicated that the cloned gene resides on YACs 1372 and 1334, placing it between *tgl* and 442 on the physical map (Fig. 6).

### *The cloned gene has RelA function*

Because ORF 2 displays sequence similarity to both RelA and SpoT proteins, a functional assay was required to indicate its role. For this purpose, we tested the *M. xanthus* clone for its capacity to complement an *E. coli relA* deletion strain to produce (p)ppGpp. Plasmid pMS381, which contains the *M. xanthus* gene under the transcriptional control of the IPTG inducible *tac* promoter, was introduced into a Δ*relA E. coli* strain, CF165 (Xiao et al. 1991). Cells carrying either pMS381 or pTTQ18 were assayed for their ability to produce (p)ppGpp in response to IPTG. Cells carrying pMS381 were able to produce (p)ppGpp in an IPTG-dependent fashion (Fig. 7), demonstrating that expression of the *M. xanthus* gene correlates with an increase in the production of this nucleotide. Strains carrying the control plasmid pTTQ18 showed no increase in (p)ppGpp levels (Fig. 7). Previously, the overproduction of *E. coli relA* in both *E. coli* (Schreiber et al. 1991) and *M. xanthus* (Singer and Kaiser 1995) have been shown to increase the level of (p)ppGpp.

### *Disruption of* relA *prevents the accumulation of (p)ppGpp in response to amino acid starvation and causes an early developmental arrest*

To assess the role of *relA* in (p)ppGpp synthesis and development in *M. xanthus,* the gene was disrupted by insertional mutagenesis. An internal 1.2-kb *Sac*I fragment (Fig. 4A) cloned to the $Km^r$ plasmid pBGS18, designated pMS300, was integrated at the chromosomal locus by homologous recombination. Integration of the plasmid at the chromosomal *relA* locus would result in a tandem duplication of the 1.2-kb region and generate two truncated genes. The structure of the expected tandem duplication was confirmed by Southern blot analysis (data not shown) and the resulting strain was designated MS10.

This *relA* insertion mutant, MS10, along with its isogenic parent, DK101, were assayed for their ability to synthesize (p)ppGpp in response to amino acid deprivation. Under these conditions DK101 accumulates (p)ppGpp within the first 15 min of starvation and the levels slowly decay (Fig. 8), consistent with previous studies (Manoil and Kaiser 1980a; Singer and Kaiser 1995). In contrast, strain MS10 does not accumulate (p)ppGpp even after 60 min (Fig. 9).

Previously, we have proposed that for the purpose of initiating fruiting body development, *M. xanthus* senses its nutritional status by measuring its intracellular levels of (p)ppGpp and thereby monitoring its capacity for protein synthesis. It follows that cells unable to produce (p)ppGpp will be unable to initiate development. DK101 and MS10 strains were examined for the ability to form fruiting bodies and spores. No fruiting bodies, mounds, or aggregates were obtained from the MS10 *relA* insertion strain, even after 5 days. Wild-type cells form fruiting bodies within 24 hr. Furthermore, when spore assays were performed after 24, 72, and 120 hr, the *relA* insertion strain produced <0.01% of the wild-type viable spore level.

Because *dfrA* lies 72-bp downstream to *relA,* it was

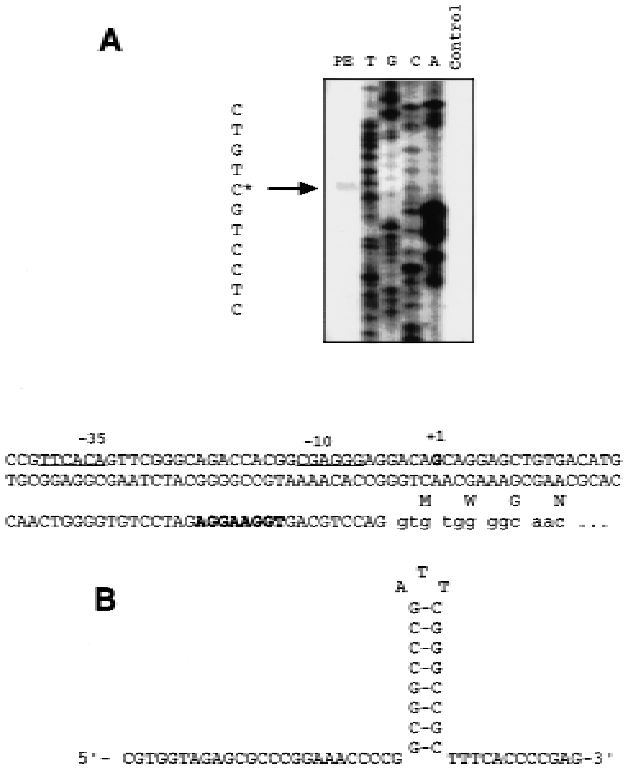


**Figure 5.** (*A*) Nucleotide sequence of the 5′-region of *relA*. Transcriptional start site for *relA* is indicated by +1 and bold caps. The −10 and −35 regions are underlined, and the putative ribosome binding site is indicated by bold caps. (PE) Primer extension product; (T, G, C, A) DNA sequence reactions with the same primer used in the primer extension analysis as indicated in Materials and Methods; (Control) tRNA control primer extension reaction. (*B*) Nucleotide sequence of the intergenic region between *relA* and *dfrA*. A putative 9-bp stem 3-bp loop structure, which may act as a transcriptional terminator, is shown.

possible that these two genes constituted a single operon and that the insertion into the upstream *relA* gene also inactivated *dfrA*. Two approaches were taken to demonstrate that the developmental phenotype associated with MS10 is caused by a disruption of *relA* and not the result of a polar effect on the downstream *dfrA* gene. In the first approach, we disrupted the intercistronic region between *dfrA* and *relA* genes by inserting plasmid pMS323 into DK101. This plasmid contains a 1-kb *Sac*I–*Nco*I fragment carrying the 3′-end of *relA* and 5′-end of the *dfrA* gene (Fig. 4A). Integration of this plasmid would duplicate this region, producing one complete *relA* gene and one complete *dfrA* gene that are separated by vector sequences. If *dfrA* is expressed from the *relA* promoter, the only intact *dfrA* gene would now be separated from its promoter. Alternatively, if *dfrA* is being expressed from its own promoter or is not essential for development, no developmental phenotype should be observed. The resulting strain, designated MS11, was assayed for development along with strains MS10 and DK101. MS11 developed and sporulated normally (unlike MS10), suggesting that either *dfrA* is not required for development or *dfrA* expression is independent of *relA* expression.

The second approach was to demonstrate that the *M. xanthus relA* gene was sufficient to rescue the developmental defect of strain MS10 when expressed in trans. For this purpose, we introduced a second copy of the *relA* gene into strains MS10 and DK101 and designated these strains MS12 and MS13, respectively. Plasmid pMS321 contains the intact *M. xanthus relA* gene and the first 130 bases of the *dfrA* gene cloned into the tetracycline resistance Mx8*att* vector, pSWU29. When assayed for development, strain MS13 regained the ability to form fruiting bodies and could sporulate to 80% of wild-type levels, demonstrating that the defect of MS10 is caused by the disruption of *relA* and not caused by polar effects on *dfrA*.

### *The* M. xanthus relA *gene rescues the DK527 mutant*

Previously, we demonstrated that the *E. coli relA* gene could rescue the developmental defect of DK527. To determine if the developmental block of strain DK527 could be rescued by the *M. xanthus relA* gene, two plasmids were constructed containing the *M. xanthus relA* gene. Plasmid pMS325 also contains Mx8*attP*, allowing us to test complementation when the *M. xanthus relA*$^+$

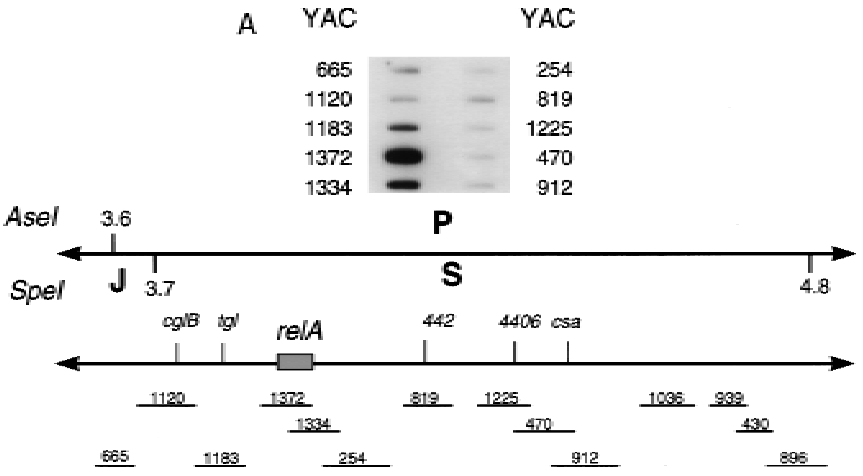


**Figure 6.** Physical mapping of the *relA* gene. (*A*) Slot blots using total YAC DNA from an ordered YAC library, probed with DNA fragment harboring the *M. xanthus relA* gene. (*B*) Schematic of the 3.6- to 4.8-Mb region of the physical map of *M. xanthus*. YACs covering this region are indicated and labeled at the *bottom*. The position of *relA* is designated by the shaded box. The *top* line represents *Ase*I and *Spe*I fragments as designated by Chen et al. (1990); the second line represents the position of previously mapped markers (Shimkets 1993).

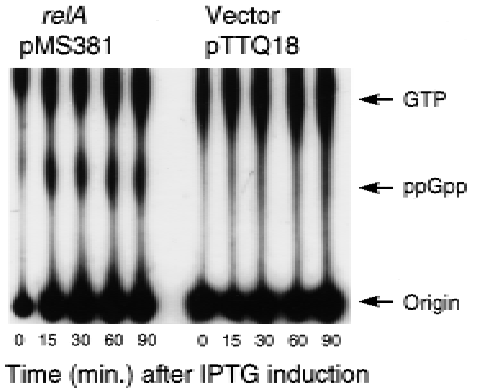


**Figure 7.** Expression of *M. xanthus relA* in *E. coli* leads to (p)ppGpp production. One-dimensional thin layer chromatography autoradiographs of extracts from *E. coli* strains carrying either pMS381 (*M. xanthus relA*) or pTTQ18 (vector), with equal radioactivity loaded at the origin in each lane.

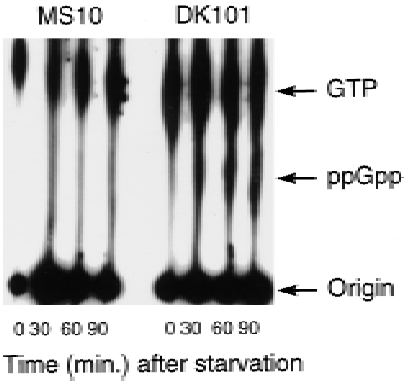


**Figure 8.** Disruption of the *M. xanthus relA* gene prevents the accumulation of (p)ppGpp. One dimensional thin layer chromatography autoradiograph of extracts from *M. xanthus* strains harboring either pMS300 (MS10) or no insertion (wild type, DK101), with equal radioactivity loaded at the origin.

gene is introduced at the *Mx8attB* site. Plasmid pMS302 lacks an *Mx8attP* site, but can integrate by homologous recombination in the *relA* gene. This would result in a tandem duplication of the *relA* region. When these plasmids were introduced into DK527, both plasmid-carrying strains were found to regain the ability to produce fruiting bodies (the pMS325-carrying strain is shown in Fig. 9) and sporulate based on direct spore count (data not shown) and viable spore assay (Table 2). Control plasmids lacking the *M. xanthus relA* gene were unable to rescue the developmental defect of DK527. These data strongly suggest that DK527 has a mutant allele of *relA*.

### *(p)ppGpp production is required for A-factor production*

One of the earliest responses to starvation is the production of an extracellular population density signal, *Myxococcus* A-factor (Kuspa et al. 1986). Because the DK527 mutant fails to express the A-factor-dependent fusions Ω4521 or Ω4400 (Fig. 3), we suspected that DK527 might be deficient in A-factor production. To examine the relationship between (p)ppGpp accumulation and A-factor production, we measured the amount of A-factor produced by DK527 and the *relA* insertion mutant. Two methods were used to collect A-factor: extracellular complementation (an in situ method in which whole cells are the A-factor producers), and collection of conditioned medium that serves as the source of A-factor.

The bioassay for A-factor employs an A-signaling defective tester strain (DK4324, *asgB* Ω4521, Kuspa et al. 1986). Six different A-factor donor strains—DK101 (wild-type, positive control), DK527, DK10528 (*E. coli relA*$^+$), DK10529, MS10 (the *relA* insertion null), and *asgB* mutant DK480 (negative control) were each mixed with DK4324. In accordance with earlier experiments (Kuspa and Kaiser 1989; Kuspa et al. 1986), DK101 (*asgB*$^+$) was able to rescue the A-signaling defect of strain DK4324, whereas the *asgB480* (A-factor defective) mutant strain could not (Table 3). Strains DK527 and MS10 had significantly less ability to rescue the defect of the tester strain (Table 3), estimated as 16% and 7% of wild-type A-factor activity, respectively. The *E. coli relA*$^+$ plasmid restored to DK527 the ability to produce A-factor to 78% of the wild-type level. DK10529 produced the same level of A-factor as DK10528 (within experimental error).

A-factor production was also tested by bioassay of the conditioned medium: MC7 suspension buffer after cells had been agitated and starved therein. MC7 buffer from each of the strains described above—DK101, DK527, DK10528, MS10, and DK480—was assayed for A-factor activity at 3, 6, and 9 hr postinitiation (Table 3). Consistent with the in situ assays, DK101 produced and released A-factor, whereas DK480, DK527, and MS10 pro-

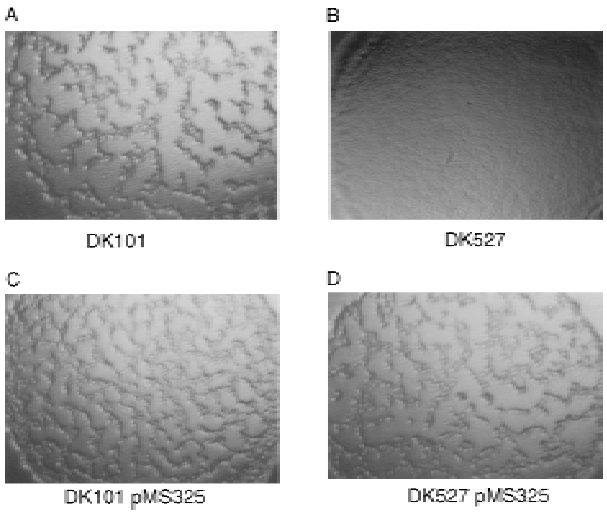


**Figure 9.** Rescue of *M. xanthus* DK527 fruiting body formation by wild-type *M. xanthus relA*. Strains DK101 and DK527 with no plasmid (*A*,*B*) or with pMS325 (*M. xanthus relA* integrated at the Mx8*att* site) (*C*,*D*) were spotted on TPM agar as described in Materials and Methods and photographed at 72 hr poststarvation.

**Table 2.** *Sporulation in DK527 rescue strains*

| Strain | Percent DK101 viable spores (± S.D.) | | |
|---|---|---|---|
| | 72 hr (3 days) | 120 hr (5 days) | 168 hr (7 days) |
| DK101 (wild type) | 100 (±14.0) | 100 (±2.7) | 100 (±1.7) |
| DK527 (*relA527* mutant) | 0.0007 (±0.0002) | 0.0002 (±0.00009) | 0.0002 (±0.0001) |
| MS16 (DK101 pMS325) | 84.8 (±10.4) | 71.9 (±5.6) | 78.6 (±2.5) |
| MS17 (DK527 pMS325) | 109.4 (±15.3) | 107.1 (±5.7) | 102.1 (±7.0) |

duced only 2%, 8%, and 3% of wild-type A-factor activity after 3 hr. Not only were DK527 and MS10 deficient for A-factor production at 3 hr, normally the time of peak level of A-factor production (Kuspa et al. 1992), but there was no evidence for delayed production of A-factor at 6 or 9 hr postinitiation (Table 3). Finally, the DK10528 strain recovered the ability to produce A-factor and to release it into the medium. The data of Table 3 shows that DK527 is defective in the production of A-factor and that the defect can be rescued by expression of the *E. coli relA*$^+$ gene in DK527.

## Discussion

When faced with starvation, *M. xanthus* has a choice between two responses to that stress: It can either slow its rate of growth to one that can be sustained at the nutrient level of its surroundings, or it can initiate fruiting body development. What role might (p)ppGpp have in that decision? As shown previously, expression of the *E. coli relA*$^+$ gene in *M. xanthus* leads to accumulation of (p)ppGpp and activation of early developmental gene expression, even in the presence of a nutrient level sufficient to support growth (Singer and Kaiser 1995). Thus, accumulation of (p)ppGpp appears to be sufficient to initiate the developmental process and represents nutrient limitation.

Previously, we showed that ectopic production of (p)ppGpp in *M. xanthus* is sufficient to activate the A-factor-dependent Ω4521 fusion in the presence of nutrients (Singer and Kaiser 1995). However, (p)ppGpp cannot activate the *asgB480* mutant. Thus both (p)ppGpp and

**Table 3.** *A-factor production*

| Donor strain | In situ production[a] | | Production in suspension[a] | | |
|---|---|---|---|---|---|
| | U/ml | %W.T. | time[b] | U/ml | %W.T.[c] |
| DK101 (wild type) | 480 ± 86 | 100 | 3 (hr) | 36 ± 3.8 | 100 |
| | | | 6 (hr) | 29 ± 3.2 | 100 |
| | | | 9 (hr) | 22 ± 3.1 | 100 |
| DK480 (DK101 *asgB*) | 28 ± 12 | 6 | 3 (hr) | 0.8 ± 0.3 | 2 |
| | | | 6 (hr) | 1.1 ± 0.4 | 4 |
| | | | 9 (hr) | 1.2 ± 0.4 | 5 |
| DK527 (DK101*relA527*) | 76 ± 22 | 16 | 3 (hr) | 2.2 ± 0.8 | 8 |
| | | | 6 (hr) | 2.3 ± 0.9 | 7 |
| | | | 9 (hr) | 2.2 ± 0.8 | 10 |
| MS10 (DK101*relA*::pMS300) | 35 ± 08 | 7 | 3 (hr) | 1.1 ± 0.2 | 3 |
| | | | 6 (hr) | 0.9 ± 0.4 | 3 |
| | | | 9 (hr) | 0.8 ± 0.4 | 4 |
| DK10529[d] (DK101 pMS132) | 425 ± 73 | 88 | 3 (hr) | 39 ± 4.8 | 108 |
| | | | 6 (hr) | 32 ± 4.2 | 110 |
| | | | 9 (hr) | 21 ± 3.1 | 95 |
| DK10528[d] (DK527 pMS132) | 375 ± 76 | 78 | 3 (hr) | 30 ± 4.6 | 83 |
| | | | 6 (hr) | 26 ± 4.8 | 89 |
| | | | 9 (hr) | 23 ± 4.8 | 104 |

[a]Values are shown as units per milliliter of cells (or per milliliter of supernatant recovered from shaken suspensions) at a cell density of 1000 Klett units, prepared as described in Materials and Methods. The values given are an average of three independent experiments.
[b]Time values indicate that the duration cells were cultured in MC7 buffer prior to bioassay of A-factor.
[c]%W.T. activity was calculated by comparing the A-factor activity found in DK480 and DK527 to the amount found in the wild-type strain. Wild-type activity was considered 100% for each time point.
[d]Because levels of (p)ppGpp adequate for fruiting body development are produced by these strains, experiments were performed in the dark, under conditions that lead to fruiting body formation for these strains.

*asgB*$^+$ are required for activation of Ω4521. Because neither DK527 nor the *relA* insertion strain is able to produce (p)ppGpp, (p)ppGpp may work through *asgB* (and/or *asgA* and *asgC*) to initiate A-factor production. Were there a second or alternative starvation sensor as well as (p)ppGpp, then this hypothetical sensor might allow starvation-induced A-factor production even in the absence of (p)ppGpp accumulation. However, little A-factor was produced by either strain unable to produce (p)ppGpp, whereas restoration of (p)ppGpp production by the *E. coli relA*$^+$ largely restored A-factor production. These data are consistent with a model in which individual cells monitor their nutritional status by means of changes in the intracellular concentration of (p)ppGpp, such that accumulation of this nucleotide initiates the developmental program. One output of the early parts of the program would be the activation of A-factor production through the *asgA, asgB,* and *asgC* gene products.

Consistent with the suggestion that (p)ppGpp works through *asg* gene products is the recent work of Plamann and colleagues, showing that *asgC* is the *M. xanthus* homolog of *rpoD,* the *E. coli* gene that encodes the $\sigma^{70}$ subunit of its RNA polymerase (Davis et al. 1995). The *asgC767* mutation, which almost completely eliminates A-factor production (Kuspa and Kaiser 1989), results from a glutamic acid to lysine change at amino acid position 598 in the *M. xanthus* $\sigma^{70}$ homolog (Davis et al. 1995). This homolog is called SigA in *M. xanthus* (Inouye 1990). Glutamate 598 is adjacent to an equivalent amino acid position in the *E. coli* $\sigma^{70}$ protein, at which suppressors of a Δ*relA* Δ*spoT* double-null mutation arise (Hernandez and Cashel 1995). Experiments with *E. coli* mutants carrying either the *rpoD*(P504L) allele or the *rpoD*(S506) mutation suggest that region 3 of the *E. coli* $\sigma^{70}$ subunit may be directly involved in (p)ppGpp-mediated gene regulation (Hernandez and Cashel 1995).

Experiments reported here combined with those of Singer and Kaiser (1995) imply that the accumulation of (p)ppGpp in response to nutrient deprivation is both necessary and sufficient to activate the developmental program of *M. xanthus.* Moreover, intracellular production of (p)ppGpp precedes, and is necessary for, extracellular production of A-factor. The next challenge is to understand how (p)ppGpp and A-factor jointly regulate Ω4521 and other genes that are both starvation and A-factor dependent, and how the critical choice between slow growth and initiation of fruiting body development is made.

## Materials and methods

### *Bacterial strains, phage, and plasmids*

All strains and plasmids used are listed in Table 1. The reference strain used for these studies was DK101 (Manoil and Kaiser 1980a), a developmentally competent *M. xanthus* strain that contains an *sglA1* allele, allowing cells to grow well dispersed in liquid culture (Hodgkin and Kaiser 1979). For simplicity, DK101 has been referred to as wild-type throughout the paper. The isolation of DK527 from DK101 has been described (Manoil and Kaiser 1980a). The transducing mxyophages Mx4*ts18ts27hrm* (Campos et al. 1978) and Mx8*clp2* (Martin et al. 1978) have been described, as well as the *E. coli* strain MG1655 and the isogenic Δ*relA*$^+$ strain (Singer et al. 1991). The Tn*5lac* transcriptional fusions Ω4408, Ω4455, Ω4400, and Ω4521 have also been described previously (Kroos et al. 1986). DK101 and DK527 derivatives harboring each fusion were constructed by Mx8- or Mx4-mediated transduction. The presence of a single Tn*5* element in the *M. xanthus* chromosome was confirmed by Southern blotting.

### *Plasmid construction*

The 4.8-kb *Pst*I fragment encoding the *M. xanthus relA* was cloned from cosmid $G_4$ into the *Pst*I sites of pBGS18, giving rise to pMS302, and into the *Mx8attP* containing vector pPLH343, giving rise to pMS350. Subclones containing small fragments of pMS302 were cloned into either pBGS18 or pBluescriptSK (Stratagene, La Jolla, CA) by use of standard cloning protocols (Sambrook et al. 1989) and are listed in Table 1.

Plasmid pMS321 was constructed by digesting pMS302 with *Nco*I and blunting the end with T4 DNA polymerase, then digesting with *Bam*HI. This fragment was then cloned into the *Bam*HI–*Sma*I sites of pSWU29, a plasmid that carries the *Mx8attP* region and tetracycline resistance. This plasmid removes the downstream *dfrA* ORF from the original insert.

Plasmid pMS325 was constructed by digesting pMS321 with *Hin*dIII and blunting the end with DNA polymerase Klenow fragment, then by cutting with *Xho*I. This procedure removed all *M. xanthus* sequences upstream to the internal *Xho*I site of *relA*. Next, a 480-bp fragment containing 5′ sequences from the upstream *Eag*I site (blunted by DNA polymerase Klenow fragment) to the internal *Xho*I site was inserted. The final construct, which removes upstream and downstream ORFs, was verified by sequencing with an internal primer.

Plasmid pMS381, containing the *M. xanthus relA* gene under the control of the *E. coli tac* Promoter (P*tac*), was constructed in a two-step procedure. First, a 477-bp PCR fragment was isolated with primers RELA2nd06 (5′-AGTTCTCCGCCTGCTTCTCC-3′) and RelABam-1 (5′-TAGGATCCGGGTCAACGAAAGCG-AACGCA-3′), placing a *Bam*HI restriction site 20 bp from the GTG translational start. This fragment contains the 5′ end of the *relA* gene without its promoter. The resulting 477-bp PCR product was then digested with *Xho*I and *Bam*HI, giving rise to a 267-bp fragment encoding the 5′ end of the *relA* gene. The 267-bp *Xho*I–*Bam*HI fragment was then cloned into pMS302 and designated pMS380. The construct was verified by sequencing with an internal primer. To construct pMS381, the 3.3-kb *Bam*HI–*Hin*dIII fragment from pMS380 was cloned into the *Bam*HI–*Hin*dIII site of pTTQ18 (Amersham, Arlington Heights, IL), placing *M. xanthus relA* under the control of P*tac.* The junction fragment was confirmed by DNA sequencing.

### *Growth and development*

*M. xanthus* cells were grown in either CTT liquid [1% casitone (Difco Laboratories), 10 mM (Tris-H)ydrochloride (pH 7.6), 1 mM $KHPO_4$ (pH 7.8), 8 mM $MgSO_4$], M1 liquid (Zusman et al. 1971), or A1 liquid (Bretscher and Kaiser 1978) at 33°C with vigorous agitation. For solid support, agar to a final concentration of 1.5% (wt/vol) (CTT and M1), or agarose to a final concentration of 0.8% (wt/vol) (A1) was added as described (Bretscher and Kaiser 1978). Where indicated, medium was supplemented with oxytetracycline (12 μg/ml) or kanamycin (40 μg/ml). Cell growth in liquid cultures was monitored with a Klett–Summerson photoelectric colorimeter (model 800-3) equipped with a red filter; 100 Klett units corresponds to $5 \times 10^8$ cells/ml. *E. coli* cells were grown in M9 complete medium (Singer et al. 1991). For

RNA synthesis experiments with serine hydroxamate and for labeling with [$^3$H]uridine, *E. coli* cells were grown in M9 complete medium lacking serine and uracil (Singer et al. 1991).

Development was initiated in *M. xanthus* by starvation as described previously (Kroos et al. 1986). Developmental β-galactosidase assays were performed as described (Kroos et al. 1986). The time of initial β-galactosidase expression (expression time) was determined by extrapolating the curve of increasing enzyme activity to its intercept with a line from the $t = 0$ value prolonged parallel to the time axis (Kroos et al. 1986). Fruiting body development was monitored visually with a dissecting microscope (Wild-Heerbrug, Switzerland). Two methods were used to examine sporulation efficiency. First, spore viability assays were performed as described previously (Thony-Meyer and Kaiser 1993). Second, direct spore counts were performed with a Petroff–Hausser counting chamber and phase contrast microscopy.

### *Light induction of* E. coli *RelA in* M. xanthus

*M. xanthus* strains were grown in liquid or on solid support medium and irradiated as described previously (Singer and Kaiser 1995).

### *PCR analysis*

Two synthetic primers, R972 [5′-AAAAAGAATTCAACGG(C/G) TACCAG(T/A)(C/G)(C/G)ATCCACAC-3′] and X1122 [5′-AAAAAATCTAGA(C/G) CCCTT(C/G)GG(C/G)GTGAA(C/G)-ACGTA-3′], were used to amplify an internal segment of the *M. xanthus relA* homolog from strain DK101 by the polymerase chain reaction. Reaction mixtures contained 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each of the four dNTPs, 2.5 units of *Taq* polymerase, 40 pmole of each primer, and 50 ng of chromosomal DNA in a final reaction volume of 100 μl. Samples were denatured at 95°C for 2 min and subjected to 30 cycles of denaturation (95°C for 1 min), annealing (57°C for 30 sec), and extension (72°C for 1 min) with a final extension incubation for 10 min, at 72°C. PCR products were then analyzed by agarose gel electrophoresis (1.5% wt/vol). The resulting 311-bp fragment was isolated by use of the QIAEX II gel extraction kit (Qiagen Inc., Chatsworth, CA) and cloned into the *Eco*RI and *Bam*HI sites of pBGS18.

### *Colony hybridization and radioactive probe protocols*

Nitrocellulose filters containing an ordered array of DNA from an *M. xanthus* cosmid library, were provided by R. Gill. Filters were probed by use of the 311-bp PCR-generated DNA fragment labeled with $^{32}$P. Radioactive probes were made by the random primer method (Sambrook et al. 1989) and [α-$^{32}$P]GTP (NEN) at 3000 Ci/mmole, 10 mCi/ml.

### *Total RNA synthesis*

RNA synthesis rates in vivo were measured as described previously (Singer et al. 1991) with the following modifications. Cells were grown in either M9 glucose (*E. coli*) or M1 (*M. xanthus*) medium to a density of $5 \times 10^8$ cells/ml, then serine hydroxamate (final concentration 2.5 mg/ml) was added to inhibit the charging of seryl-tRNA. Portions of 100 μl were removed for analysis: two portions prior to addition of serine hydroxamate ($t$=0), followed by experimental portions (performed in duplicate) at the time intervals indicated in Figure 1. All samples were exposed for 2 min to 10 μCi/ml [5,6-$^3$H]uridine (Amersham TRK. 410; 40 Ci/mmole) plus 2 μg/ml uridine. Incorporation was stopped by addition of 2 ml of ice-cold 5% trichloroacetic acid (TCA) for 30 min. Samples were washed three times with 5 ml of ice-cold 2% TCA, rinsed with 70% ethanol, air-dried, and the $^3$H-incorporated into TCA insoluble material was determined in a Beckman scintillation counter (model LS 1801). Incorporation of radioactivity was linear for up to 30 min under these conditions.

### *Measurement of guanosine nucleotides*

Guanosine nucleotides were isolated and measured as described previously (Manoil and Kaiser 1980a; Singer and Kaiser 1995).

### *Blotting and physical mapping*

Southern blotting was performed as described previously (Sambrook et al. 1989). Physical mapping was performed by the Bio-Rad CHEF Mapping DRII system with a 1% agarose gel in 0.5% TBE buffer at 10°C for 15:10 hr at 6V/cm (200 V) with switch times of 70 and 120 sec. *M. xanthus* DNA was prepared and digested in agarose plugs as described previously (Chen et al. 1990, 1991). CHEF gels were then used for Southern blot analysis.

Gene locations were further localized on the *M. xanthus* physical map by performing Southern blot analysis by use of an ordered set of *M. xanthus* YAC clones (Kuspa et al. 1989; Chen et al. 1990, 1991). Whole yeast DNA (containing the YAC DNA) was purified from strains carrying *M. xanthus* YAC clones (Coulson et al. 1988). Samples were denatured with 0.4 M NaOH at 65°C for 15 min, and 25 μg of DNA was transferred to a Nytran filter (Amersham, Arlington Heights, IL) by use of a slot blot vacuum apparatus (Bio-Rad, Hercules, CA). The DNA was then cross-linked to the membrane with a UV transilluminator and subjected to Southern hybridization procedures as described above.

### *Western blots*

Western blots were performed as described previously (Straus et al. 1987; Singer and Kaiser 1995) Anti-*E. coli* RelA antibody was provided by G. Glaser (Hadassa Medical School, Jerusalem, Israel).

### *Sequence analysis*

DNA sequence analysis was performed by the Division of Biological Studies Automated DNA Sequencing Facility, at the University of California, Davis, with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit using AmpliTaq DNA polymerase. Reactions were run on a 4.25% acryl/bisacrylamide gel with an ABI Prism 377 DNA Sequencer. Sequence data was analyzed by use of the ABI Prism Sequencing 2.1.1 software (Perkin Elmer, Foster City, CA) and contigs assembled with Sequencher v3.0 (GeneCodes Corporation, Madison, WI).

### *Primer extension*

Vegetative RNA was isolated from DK101 cells as described previously (Kaplan et al. 1991). Primer extensions were performed as per Mirel and Chamberlin (1989) with the following modifications: 25 μg of vegetative RNA or tRNA was used in each reaction, by use of the synthetic oligonucleotides RelA2nd05 (5′-CCTTCTTGATGATGTCCAG-3′) and RelA2nd06 (5′-AG-TTCTCCGCCTGCTTCTCC-3′). Samples were run on a 5% Long Ranger (J.T. Baker, Phillipsburg, NJ) urea acrylamide gel alongside DNA sequence reactions. Sequence reactions were

performed with the fmol DNA cycle sequencing kit (Promega, Madison, WI), as described by the manufacturer. Primers used for primer extension were simultaneously used for fmol cycle sequencing reactions.

*A-factor assays*

A-factor production in situ was assayed as described previously (Kuspa et al. 1986). A-factor released into the extracellular medium by the donor cells was assayed as described (Kuspa and Kaiser 1989).

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