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MazF, an mRNA Interferase, Mediates Programmed Cell Death during Multicellular *Myxococcus* Development

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SUMMARY

In prokaryotes, the toxin-antitoxin systems are thought to play important roles in growth regulation under stress conditions. In the *E. coli* MazE-MazF system, MazF toxin functions as an mRNA interferase cleaving mRNAs at ACA sequences to inhibit protein synthesis leading to cell growth arrest. *Myxococcus xanthus* is a bacterium displaying multicellular fruiting body development during which approximately 80% of cells undergo obligatory cell lysis. Here, we demonstrate that *M. xanthus* has a solitary *mazF* gene that lacks a cotranscribed antitoxin gene. The *mazF* deletion results in elimination of the obligatory cell death during development causing dramatic reduction in spore formation. Surprisingly, MrpC, a key developmental regulator, functions as a MazF antitoxin and a *mazF* transcription activator. Transcription of *mrpC* and *mazF* is negatively regulated via MrpC phosphorylation by a Ser/Thr kinase cascade. These findings reveal the regulated deployment of a toxin gene for developmental programmed cell death in bacteria.

INTRODUCTION

While programmed cell death (PCD) pathway is a well-established eukaryotic developmental process, it is not clear if any developmental process in bacteria similarly require a well-defined PCD pathway. Obligatory cell lysis observed during *Bacillus* sporulation and *Myxobacteria* fruiting body formation exemplify forms of bacterial PCD (Lewis, 2000; Gonzalez-Pastor et al., 2003; Engelberg-Kulka and Hazan, 2003). *Myxococcus xanthus*, a unique soil Gram-negative bacterium, exhibits social behavior during vegetative growth and multicellular development forming fruiting bodies upon nutrient starvation. The developmental processes of *M. xanthus* have been shown to be regulated by a series of sophisticated intercellular signaling pathways that activate expression of a different set of genes with precise temporal patterns during development (Dworkin, 1996; Julien et al., 2000). During *M. xanthus* fruiting body formation, the majority (approximately 80%) of the cells undergo altruistic obligatory cell lysis, while the remaining 20% are converted to myxospores (Wireman and Dworkin, 1977; Nariya and Inouye, 2003). Although the exact autolysis mechanism remains obscure, *M. xanthus* contains a large number of autolysin genes encoding for enzymes that degrade the cell wall (TIGR: http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org=gmx). Curiously, however, none of these autolysin genes have been shown to be essential for developmental autolysis.

Toxin-antitoxin (TA) systems are widely found in bacterial chromosomes and plasmids. These systems generally consist of an operon that encodes a stable toxin and its cognate labile antitoxin. They form a stable complex under normal growth conditions, while under stress conditions the toxin is released from the complex to exert its toxic function as a result of antitoxin degradation. Genomic analysis of 126 prokaryotes revealed that there are at least eleven genome-encoded TA systems (MazEF, RelEB, DinJ/YafQ, YefM/YeoB, ParDE, HigBA, VapBC, Phd/Doc, CcdAB, HipAB, and $\varepsilon\zeta$) in free-living bacteria, while obligate host-associated bacteria living in constant environmental condition do not posses the TA modules (Pandey and Gerdes, 2005; Lioy et al., 2006). Based on these findings, it can be suggested that TA systems may play important roles during adaptation to environmental stresses. Among the TA systems, the MazE-MazF system is one of the best-studied systems; MazF from *Escherichia coli* has been shown to be an mRNA interferase specifically cleaving cellular mRNAs at ACA sequences to effectively inhibit protein synthesis and subsequently cell growth (Zhang et al., 2003). MazF induction in *E. coli* leads to a new physiological cellular state termed "quasi-dormancy," under which cells are fully metabolically active and are still capable of producing a protein in the complete absence of other cellular protein synthesis if the mRNA for the protein is engineered to be devoid of ACA sequences (Suzuki et al., 2005). Recently, Kolodkin-Gal et al. (2007) reported that for the *mazEmazF*-mediated cell death a pentapeptide is required, of which production is growth phase dependent.

Here, we analyzed the TA systems in the *M. xanthus* genome and found that it contains only a solitary *mazF* gene without a cognate *mazE*-like antitoxin gene. This *mazF* gene (*mazF-mx*) was found to be developmentally regulated and required for the obligatory cell death during fruiting body formation, as its deletion eliminated developmental cell death with a dramatic reduction in spore formation. Surprisingly, we observed that MrpC, an essential developmental transcription factor involved

in the regulation of *mazF-mx* expression, functions as an antitoxin for MazF-mx by forming a stable complex with MazF-mx. We further demonstrate that the expression of the MrpC-MazF system is negatively regulated at the level of transcription via MrpC phosphorylation by a eukaryotic-like Ser/Thr protein kinase cascade (Pkn8(Pkn14 kinase)-Pkn14(MrpC kinase)) (Nariya and Inouye, 2005a, 2006) under nutrient-rich growth conditions. Under these conditions, MazF forms a stable complex with a nonphosphorylated form of MrpC and the remaining free MrpC is inactivated by phosphorylation, so that MrpC transcription activator function is suppressed. We also show that MazF-mx is an mRNA interferase, which recognizes a specific five-base sequence, GUUGC in RNA, and cleaves between the two U residues. These findings uncover that a PCD cascade is involved in bacterial development, associating with a Ser/Thr protein kinase cascade, and is reminiscent of eukaryotic PCD.

RESULTS

Presence of a Solitary *mazF* Gene in the *M. xanthus* Genome

We found that in contrast to all known MazE-MazF systems in a number of prokaryotes, *M. xanthus* MazF (MazF-mx) is encoded by a monocistronic operon without any cognate antitoxin genes. Genomic analysis for the eleven known TA families using TBLASTN-Search, Pfam, and COG lists on the *M. xanthus* genomic database (TIGR) revealed the existence of a single MazF homolog (MazF-mx; MAXN1659) with no identifiable MazE homolog (Figure 1A). MazF-mx (122 aa) has 24% identity and 58% similarity to *E. coli* MazF (111 aa) (Figure 1B). The finding of such a solitary *mazF* gene appeared to be an exception to the hypothesis that the TA modules may play essential roles in bacterial cell growth during adaptation to environmental stresses by inducing a state of reversible bacteriostasis (Pandey and Gerdes, 2005). It also raises intriguing questions as to whether MazF-mx expression may be developmentally regulated and associated with developmental autolysis, and if there is an antitoxin for MazF-mx, considering the highly diverse nature of MazF antitoxins (Pandey and Gerdes, 2005).

MrpC, a Key Developmental Transcription Factor, as a Potential Antitoxin for MazF-mx

In order to identify the antitoxin for MazF-mx, we carried out a yeast two-hybrid screen using MazF-mx as a bait and an *M. xanthus* genomic library (Nariya and Inouye, 2005b). From 32 positive interactions found to associate with MazF-mx, 15 were encoded by *mazF-mx* and 17 were encoded by *mrpC*, indicating that MazF-mx forms an oligomer (dimer) and that MrpC may be a likely candidate antitoxin for MazF-mx. Interestingly, MrpC is a 248 residue protein that is a member of the CRP transcription regulator family and is chromosomally located 4.44 Mbp downstream of the *mazF-mx* gene. Importantly, the *mrpC* gene is essential for *M. xanthus* development (Sun and Shi, 2001) and is a key early-developmental transcription activator for the gene for FruA, another essential developmental regulator (Ueki and Inouye, 2003). Additionally, phosphorylation of MrpC by a Ser/Thr protein kinase cascade is also involved in the regulation of MrpC function (Nariya and Inouye, 2006). MrpC and MazF interaction can be further detected by pull-down assays using purified N-terminal histidine-tagged MrpC and nontagged MazF-mx expressed in the soluble fraction of *E. coli* cell extract (Figure 2A). Furthermore, in vegetative DZF1 cells, chromosomally encoded MazF-mx was coimmunoprecipitated with MrpC using anti-MrpC IgG (lanes 1 and 3 in Figure 2B), demonstrating that MazF-mx forms a complex with MrpC in vivo.

MazF-mx Is Essential for Obligatory Cell Death during Development

In order to elucidate the role of MazF in the life cycle of *M. xanthus*, a *mazF-mx* in-frame deletion strain (Δ*mazF*) was constructed. While vegetative growth of Δ*mazF* was normal, we observed that development was profoundly affected. When the concentrated vegetative cells at the mid-log phase (2×10^{10} cells/ml) of Δ*mazF* and the parental cells (DZF1) were spotted (5 μl; 10^8 cells) onto limited-nutrient CF agar plate, DZF1 developed normally within 48 hr forming compact fruiting bodies (FB) consisting of myxospores, while development of Δ*mazF* was delayed and compact FB were not formed resulting in very poor spore yields (at only 8% of the yield of wild-type spores; Figure 2C). Even after 72, 96, and 120 hr of development, FB of Δ*mazF* cells appeared to be very loose and relatively translucent with poor spore yield (15%, 18%, and 18%, respectively) compared to DZF1. Cell autolysis and viability during development were also examined (Figure 2D); cell numbers for both Δ*mazF* and DZF1 almost doubled cell numbers at 12 hr after spotting on CF plates. After this time point, DZF1 cell numbers dramatically decreased to 18% due to autolysis. At the 24 hr time point, the surviving wild-type cells begin to be converted to myxospores. In contrast, Δ*mazF* cell numbers only slightly reduced to 77% and were maintained at that level even at 48 hr (Figure 2D). Interestingly, DZF1 cell viability was substantially reduced (less than 1%) after 24 hr of development, while over 30% of Δ*mazF* cells were able to form colonies on CYE plates (Figure 2E). When development-defective Δ*mrpC* cells (Nariya and Inouye, 2006) were examined in a similar manner, they were found to be completely incapable of growth on CF plates (Figure 2D), while cell viability only gradually decreased in contrast to DZF1 and Δ*mazF* cells. The Δ*mrpC* cell could not form FB (Figure 2B), and the Δ*mrpC* cell viability continued to decrease (Figure 2E). A LIVE/DEAD stain technique (Invitrogen) was also applied to detect cell death during *M. xanthus* development. At 18 hr of development, approximately 63% of DZF1 cells lysed, which cannot be detected by the staining, but the remaining 37% of the cells were stainable, among which 54.3% were stained as dead cells (Figure 2F). On the other hand, only 9.2% of stainable Δ*mazF* cells were found dead (Figure 2F). These observations indicate that MazF-mx is required for developmental autolysis to achieve effective fruiting body formation and sporulation.

Developmental Regulation of *mazF-mx* Expression by MrpC

Since the expression of the *mazEF* operon in *E. coli* is negatively autoregulated by the MazE-MazF complex (Marianovsky et al., 2001), we next examined the role of MrpC in regulating *mazF-mx* expression. By primer extension (Figure 3A) using total RNAs isolated from DZF1, the transcriptional initiation

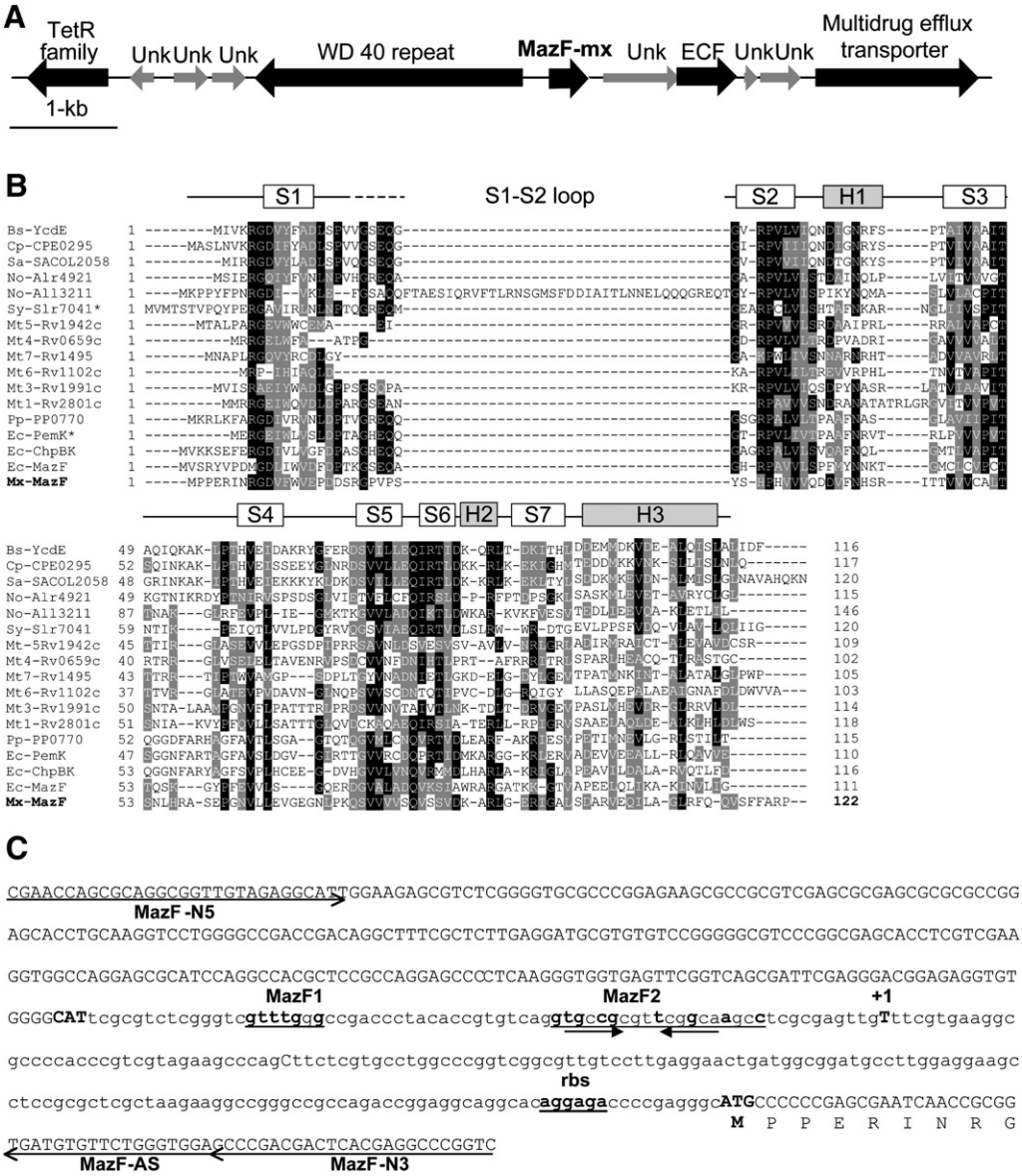

Figure 1. Location of the *mazF-mx* Gene on the *M. xanthus* Chromosome and Sequence Alignment of MazF Homologs

(A) Location of *mazF-mx* (MAXN1659; 1962661::1963026) on the *M. xanthus* chromosome was obtained from TIGR. ORF with unknown (Unk) or known function is shown with gray or black arrows; TetR family transcriptional regulator, WD40 repeats containing protein, ECF-sigma factor, and multidrug efflux transporter are also shown.

(B) Alignment of *M. xanthus* MazF (Mx-MazF) with those of *B. subtilis 168* (Bs), *C. perfringens 13* (Cp), *S. aureus COL* (Sa), *Nostoc PCC7120* (No), *Synechocystis PCC6803* (Sy), *M. tuberculosis H37Rv* (Mt1 ~7) (Zhu et al., 2006), and *E. coli K12* (Ec). β strand (S) and helical (H) regions are assigned according to Ec-MazF (Kamada et al., 2003). Amino acid residues identical are shown by black shades, and conservative substitutions by gray shades. Plasmid-borne MazF is indicated with an asterisk.

(C) DNA sequence of the *mazF-mx* promoter region. The transcription initiation site is indicated by +1. Putative MrpC binding sites, MazF1, and MazF2 are shown by bold letters. The sequences corresponding to primers used for PCR and the primer extension are underlined with arrows.

site of *mazF-mx* was localized 164 bases upstream from the initiation codon (Figure 1C) for both vegetative growth and the development phases. Notably, the level of *mazF-mx* transcript significantly increased upon nutritional starvation (Figure 3A), indicating that *mazF-mx* is developmentally induced. To further confirm this notion, a *lacZ-mazF-mx* fusion was constructed and introduced into DZF1 at the original chromosomal location. β-galactosidase assay of this constructed strain (*mazF-mx*^{P}*-lacZ*/DZF1) showed that *mazF-mx-lacZ* was expressed at approximately 20~30 U during vegetative growth and steadily increased after 6 hr at the onset of development and reached 55 U at 24 hr (Figure 3B). These results are in agreement with the results of primer-extension analysis (Figures 3A and 3E).

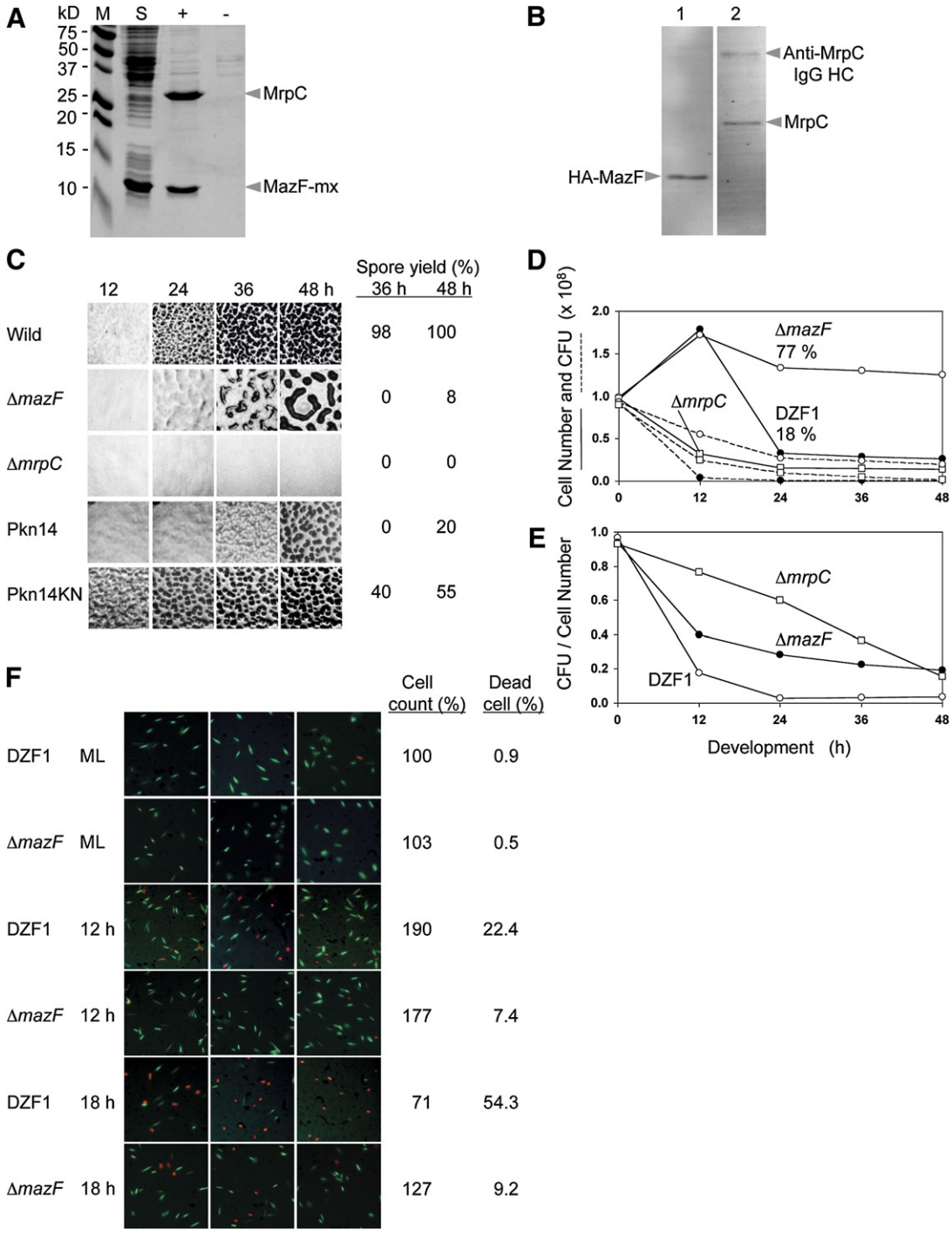

Figure 2. Roles of MazF-mx and MrpC in *M. xanthus* Development

(A) Interaction between MazF-mx and MrpC in a pull-down assay. Soluble fraction (S) from *E. coli* cells expressing nontagged MazF-mx was incubated with (+) or without (–) purified His-tagged MrpC. The complex was recovered by the nickel-resin. The positions of His-tagged MrpC and MazF-mx are shown by arrows.

(B) Interaction between MazF-mx and MrpC in vivo. MrpC was immunoprecipitated using anti-MrpC IgG from the soluble fraction prepared from DZF1 (lanes 1 and 3) and Δ*pkn14* (lanes 2 and 4) cells at the mid-log phase. Immunoprecipitates were then analyzed by western blot using mouse anti-MazF-mx serum (left panel) and anti-MrpC IgG (right panel).

(C) Developmental phenotypes on CF agar plates 12, 24, 36, and 48 hr after development. Spore yields at 36 and 48 hr are shown by considering the yield of the wild-type DZF1 spores at 48 hr as 100%. Pkn14 and Pkn14KN represent pKSAT-Pkn14/DZF1 and pKSAT-Pkn14KN/DZF1, respectively.

(D and E) Developmental analysis of the total cell numbers and colony forming units (CFU). Numbers of rod-shape cells (solid line) and CFU (dotted line) of Δ*mazF* (open circles), DZF1 (closed circles), and Δ*mrpC* (open squares) were measured in (D). The ratios of CFU to cell number were plotted in (E).

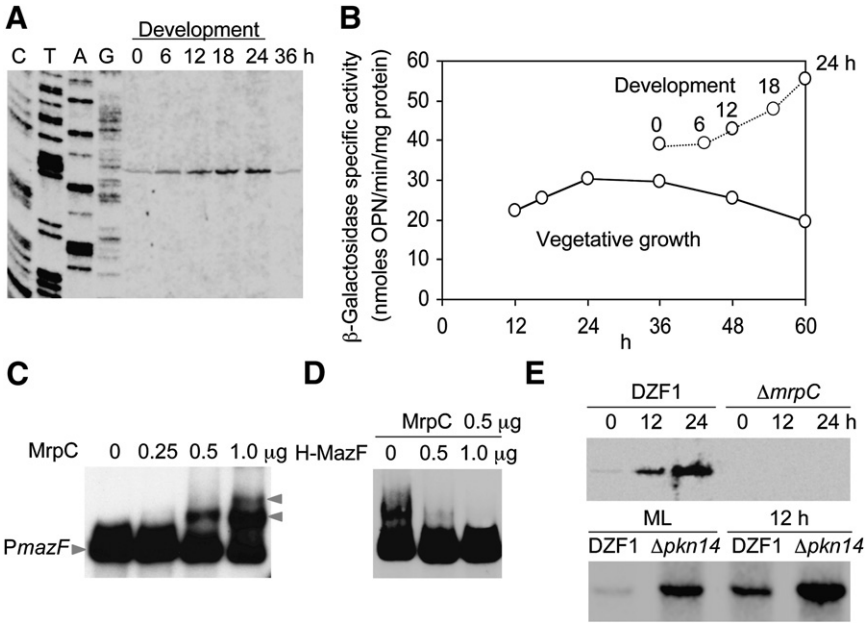

Figure 3. Expression and Regulation of the *mazF-mx* Gene during the *M. xanthus* Life Cycle

(A) Primer-extension analysis of the *mazF-mx* expression during development.

(B) β-galactosidase assay of *mazF-mx* promoter *lacZ* fusion integrated into the chromosome.

(C) Gel-shift assay of MrpC on the *mazF-mx* promoter. Arrows indicate the bands newly formed in the presence of MrpC.

(D) Gel-shift assay of MrpC preincubated with purified His-tagged MazF-mx (H-MazF) prior to gel-shift assay.

(E) Primer-extension analysis for *mazF-mx* expression was carried out using total RNA from DZF1 and Δ*mrpC* cells at 0, 12, and 24 hr in development (upper panel) and from the mid-log (ML) phase cells and 12 hr development cells of DZF1 and Δ*pkn14* (lower panel).

Next, we examined whether MrpC can bind to the *mazF-mx* promoter. Gel-shift assay using purified MrpC and the *mazF-mx* promoter region from −73 to +166 (P*mazF*; Figure 3C) showed that MrpC binds to at least two sites on the *mazF-mx* promoter region. On the basis of the consensus sequence: A/GTTTC/GAA/G and GTGTC-N_8-GACAC (N is any base; Nariya and Inouye, 2006), two MrpC-binding sites may be assigned at the regions from −56 to −50 (MazF1) and from −29 to −12 (MazF2; Figure 1C). Binding of MrpC to the promoter region was found to be inhibited when MrpC was preincubated with MazF-mx (Figure 3D). Furthermore, the *mazF-mx* expression in Δ*mrpC,* analyzed by primer extension (Figure 3E), became undetectable during both vegetative growth and the development phase, indicating that MrpC is a transcription activator for developmental *mazF-mx* expression.

MazF-mx Toxicity in *M. xanthus*

In order to detect MazF-mx toxicity in *M. xanthus*, *mazF-mx* was cloned in an *M. xanthus* expression vector, pKSAT, which can constitutively express a cloned gene during vegetative growth and the development phase. The resulting pKSAT-MazF-mx was then integrated into the chromosome by site-specific (*attB*/*attP*) recombination. Furthermore, a hemagglutinin epitope (HA)-tagged *mazF-mx* was also constructed and cloned in pKSAT (pKSAT-HA-MazF) to detect its expression in *M. xanthus* by western blot analysis. These constructs were first introduced into Δ*mazF,* resulting in the strains pKSAT/Δ*mazF* (vector control), pKSAT-MazF/Δ*mazF*, and pKSAT-HA-MazF/Δ*mazF*. No significant growth defect was observed in any of the strains during vegetative growth (Figure 4A). Note that MrpC expression level in Δ*mazF* was similar to that in DZF1 during both vegetative growth and development (not shown). The formation of the MrpC-MazF (HA-tagged) complex in pKSAT-HA-MazF/Δ*mazF* cells was detected by immunoprecipitation using anti-MrpC IgG in vivo (not shown). Importantly, the defective developmental phenotypes of Δ*mazF* were partially restored by the introduction of pKSAT-MazF, which could form compact FBs and yield myxospores at an intermediate level (Figure 4B), while the introduction of pKSAT vector alone was unable to restore the phenotypes. Notably, severe cell toxicity by MazF-mx was observed in Δ*mrpC*. While pKSAT-HA-MazF/Δ*mrpC* was able to grow in CYE medium, its growth-rate was significantly reduced and the cells could not reach to the maximum density (350 Klett) as the growth stopped at 220 Klett density (Figure 4A). Interestingly, the cells then rapidly lysed forming aggregates (to 50 Klett), while the density of control cells only gradually decreased without forming aggregates (to 220 Klett) at 72 hr. A very similar phenotype was observed with pKSAT-MazF/Δ*mrpC.* In this strain, significantly higher populations of the cells were found dead by the LIVE/DEAD stain (Figure 4C). Constitutive expression of HA-tagged MazF-mx in *M. xanthus* was confirmed by the western blot analysis using an HA antibody at the mid-log and mid-stationary phases (not shown). These results indicate that MazF-mx expression in the absence of MrpC expression is toxic, confirming the prediction that MrpC functions as an antitoxin to MazF-mx.

MazF-mx Is an mRNA Interferase and MrpC Is an Antitoxin for MazF-mx

Since MazF-mx expression did not exhibit strong cellular toxicity in *E. coli* (not shown), we speculated that MazF-mx may cleave

(F) LIVE/DEAD staining of developmental cells. 10^8 vegetative cells at the mid-log phase (ML) and developmental (12 and 18 hr) cells initiated using 10^8 vegetative cells were subjected to LIVE/DEAD stain. Stained cells were observed using a fluorescence microscopy. Twelve frame of pictures for each sample were taken to count live (green) and dead (red) cells.

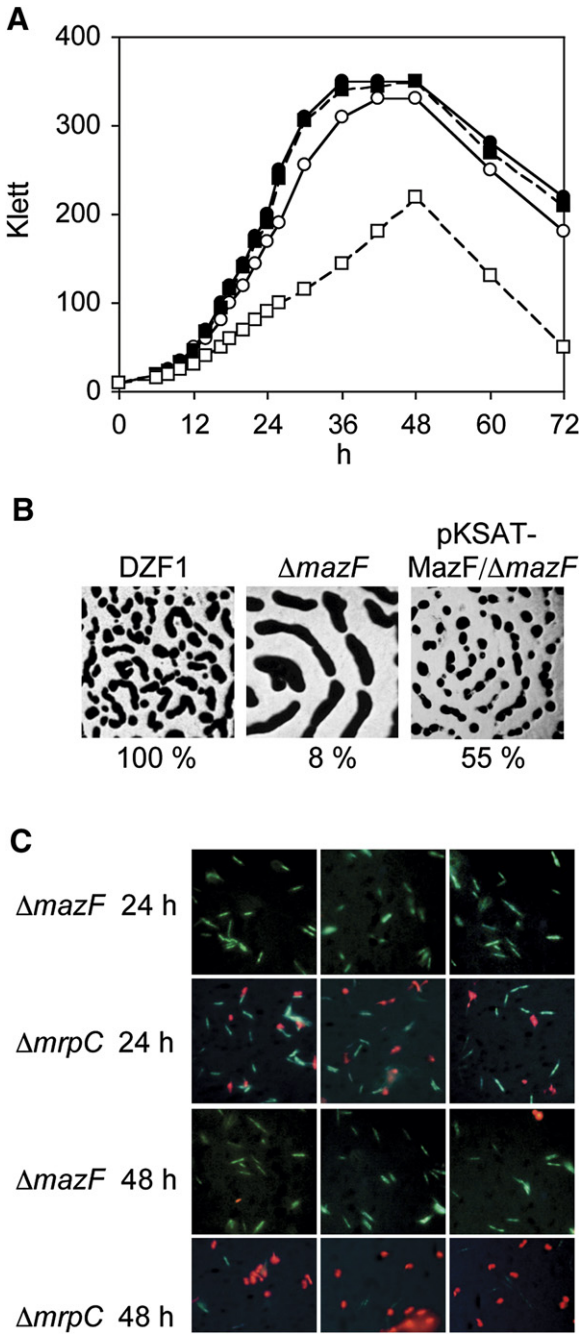

Figure 4. Growth Curves and Morphology of Δ*mazF* and Δ*mrpC* Strains

(A) Cell toxicity of MazF-mx expression during vegetative growth in Δ*mazF* and Δ*mrpC*. The cells from the deletion strains were transformed with either pKSAT-MazF-mx or pKSAT (control) and their growth curves are shown: pKSAT (filled circles) or pKSAT-HA-MazF-mx (open circles) in Δ*mazF* (solid lines) and pKSAT (filled squares) and pKSAT-HA-MazF-mx (open squares) in Δ*mrpC* (dotted lines).

(B) Development morphology of DZF1 (wild-type), Δ*mazF*, and Δ*mrpC* cells carrying pKSAT-HA-MazF-mx on CF agar plates and spore yields at 48 hr in development. The spore yield is expressed as percentage of that for DZF1.

(C) LIVE/DEAD staining of pKSAT-HA-MazF/Δ*mazF* and pKSAT-HA-MazF/Δ*mrpC* cells. 10^8 vegetative cells at 24 and 48 hr in (A) were subjected to LIVE/DEAD staining.

mRNAs at more specific sites as compared to *E. coli* MazF. Purified MazF-mx did show endoribonuclease activity against *M. xanthus* total RNAs yielding free 5′-OH group (Figure 5A). When MS2 phage ssRNA (3569 bases) was used as substrate, it was cleaved into two major bands of approximately 2.8 and 0.8 kb with many minor bands between them (Figure 5B), suggesting that MS2 ssRNA may contain a preferential cleavage site for MazF-mx. Importantly, preincubation of MazF-mx with MrpC almost completely inhibited the MazF-mx endoribonuclease activity (Figure 5C), further demonstrating that MrpC functions as an antitoxin for MazF-mx.

Next, to determine which of the two major fragments contains the 5′-end of MS2 ssRNA, the RNA labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase was incubated with MazF-mx and the 0.8 kb band was found to be derived from the 5′-end (not shown). Thus, we designed four primers at positions 1337, 1078, 0660, and 0252 from the 5′ end of MS2 ssRNA to identify the cleavage site. As shown in Figure 5D, the amount of the full-extended products decreased with primers 1337 and 1078 as the amount of MazF-mx was increased in the reaction, whereas no significant change was observed with primers 0660 and 0252, indicating that the preferential cleavage site exists between positions 0660 and 1078. Subsequently, the primer-extension analysis by primer 1337 was carried out with different amounts of MazF-mx to identify the exact cleave site (Figure 5E). At a low concentration of MazF-mx (0.1 μg; lane 2), a preferential cleavage was observed at the 3′ end of the U-0724 residue (GAGU!UGCA; ! indicates the cleavage site), which yields a 0.8 kb fragment. Preincubation of MazF-mx (0.1 μg) with MrpC (0.5 μg) resulted in complete inhibition of the cleavage (lane 5). When higher concentrations of MazF-mx (lanes 3 and 4) were used, new cleavage sites appeared at 1087 (AUGU!CAGG), 1106 (ACGU!AAUA), and 1241 (ACGU!AAAG) with several other minor cleavage sites that were detected after prolonged autoradiography of the gel (Table 1). A secondary structure of MS2 ssRNA predicted by MFOLD program (Zuker, 1989; http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html) is shown in Figure 5F in which major cleavage sites are indicated by arrows. Interestingly, all cleavage sites are located on single-stranded regions, consistent with the previous finding that *E. coli* MazF cleaves only single-stranded RNAs (Zhang et al., 2003). From the alignment of all cleavage sites (Table 1), the most preferred cleavage sequence for MazF-mx is GU!UGC, in which the first G residue may be replaced with A residue.

Since MazF endoribonuclease activity has been shown to be inhibited by a moderate salt concentration and does not require metal ions (Pellegrini et al., 2005), we examined the effect of NaCl and $MgCl_2$ on MazF-mx endoribonuclease activity using a synthetic 14 nt oligoribonucleotide, MS2-0724 (UUGGAGU!UG CAGUU). When 5′-end γ-^{32}P-labeled MS2-0724 (0.01 pmole) was incubated with MazF-mx (50 ng) for 30 min at 30°C in 20 μl of MazF buffer without NaCl, the substrate was completely cleaved; however the cleavage reaction was found to be very sensitive to NaCl and $MgCl_2$ at higher than 100 and 25 mM, respectively (not shown). This salt sensitivity of MazF-mx was found to be quite useful for purification of MazF-mx because a very high expression of MazF-mx in *E. coli* can be achieved in LB medium with high salt concentrations without coexpressing

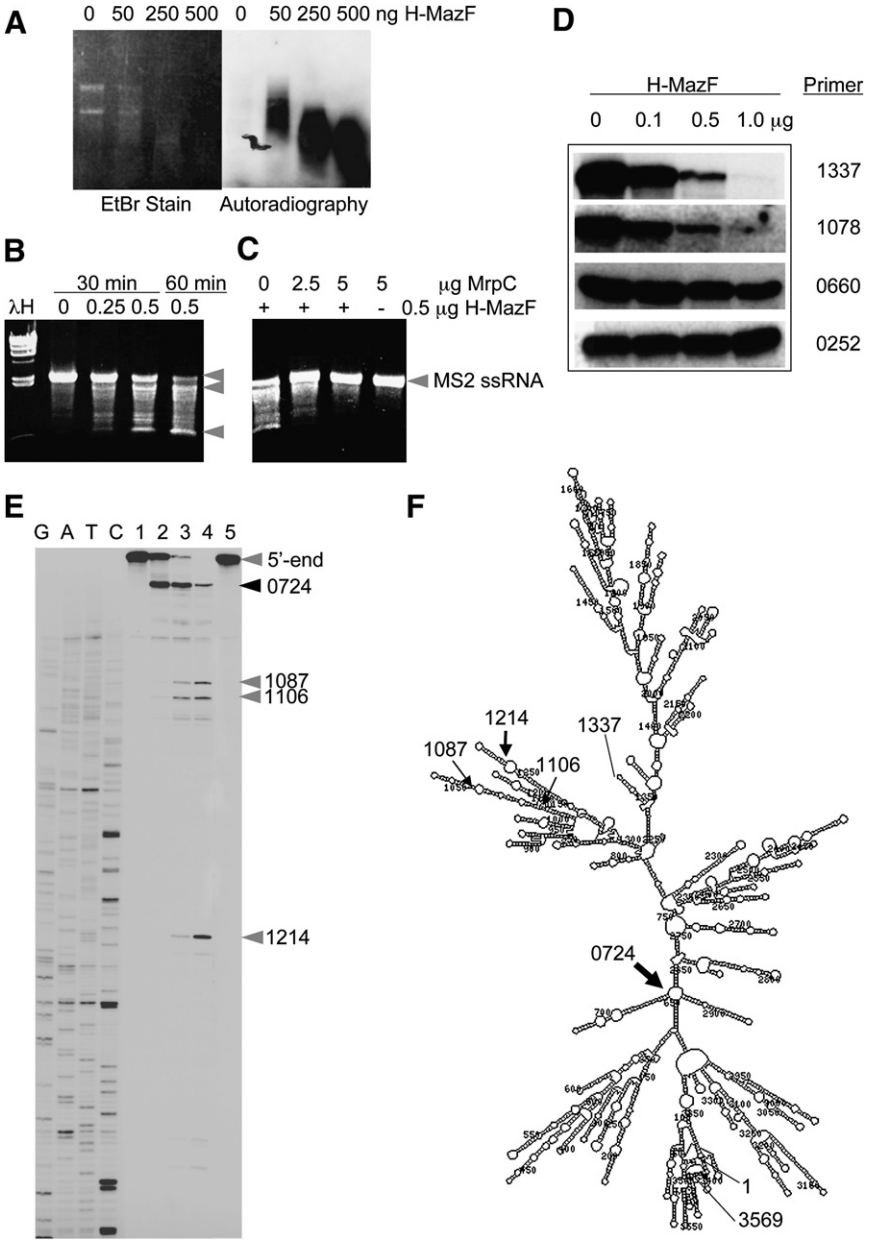

Figure 5. Endoribonuclease Activity of MazF-mx In Vitro

(A) Cleavage of *M. xanthus* total RNA by His-tagged(H)-MazF. The products were 5′-end labeled with [γ-^{32}P]ATP by T4 kinase and separated on agarose gel. The gel was stained with ethidium bromide (EtBr) (left panel). Subsequently the gel was dried and then subjected to autoradiography (right panel).

(B and C) Cleavage of MS2 ssRNA and its inhibition by MrpC. The gel was stained with EtBr.

(D) Primer-extension analysis of MS2 ssRNA digested with MazF-mx. Fully extended (5′-end) products were shown (see also E). After MS2 ssRNA (0.8 μg) was incubated with 0 (lane 1), 0.1 (lane 2), 0.5 (lane 3), and 1.0 μg (lane 4) of H-MazF in 20 μl of MazF buffer, 5 μl of sample was heated at 95°C for 1 min with 1 pmole (1 μl) of the 5′-end-labeled primer and then placed on ice for 5 min. RT reaction was carried out at 47°C for 30 min as described in Experimental Procedures. The number for each primer indicates the position of the 5′-end residue in MS2 ssRNA.

(E) Primer-extension analysis (D) used the 1337 primer and the cleavage sites by MazF-mx. Lane 5 is preincubated 0.1 μg of H-MazF (lane 1) with 0.5 μg of antitoxin, MrpC. The number indicates the position in MS2 ssRNA cleaved by H-MazF-mx.

(F) Predicted secondary structure of MS2 ssRNA by the MFOLD program (Zuker, 1989) and the corresponding cleavage sites by H-MazF in (E).

its antitoxin, as even low MazF-mx expression was lethal in the absence of salt (not shown). Interestingly, a severe developmental defect has been observed on CF plates containing NaCl at 100∼150 mM (Kimura et al., 2002), which may be at least partially due to the salt sensitivity of MazF-mx.

A Protein Kinase Cascade Negatively Regulates the MrpC-MazF System

During vegetative growth, MrpC is reported to be phosphorylated by a eukaryotic-like Ser/Thr protein kinase cascade that suppresses MrpC function to prevent untimely switch-on of the early developmental pathway (Pkn8(Pkn14 kinase)-Pkn14(MrpC kinase) cascade) (Nariya and Inouye, 2006). The genetic disruption of the Pkn8-Pkn14 cascade causes upregulation of *mrpC* resulting in acceleration of FB formation but with less spore yield (Nariya and Inouye, 2005a). We, therefore, examined the effect of MrpC phosphorylation on the MrpC-MazF system. In the *pkn14* mutant (Δ*pkn14*), the *mazF-mx* expression was also shown to be upregulated as in the case of *mrpC* expression during both vegetative growth and development (Figure 3E), and as expected a higher accumulation of the MrpC-MazF complex was detected (Figure 2B). Furthermore, it has been shown that the Pkn8-Pkn14 cascade is functioning mainly during vegetative growth (Nariya and Inouye, 2005a). As shown in Figure 2C, constitutive expression of Pkn14 in DZF1 using pKSAT resulted in delayed development, while expression of Pkn14KN, which is unable to phosphorylate MrpC, in DZF1 caused faster development as previously shown with Δ*pkn14* (Nariya and Inouye, 2005a) (see Figure 2C), probably because Pkn14KN forms inactive heterotetramers with wild-type Pkn14 produced in DZF1.

Furthermore, we also examined the effect of phosphorylation of MrpC on its ability to form the complex with MazF. For this purpose, we tested how the pretreatment of MrpC with Pkn14 affects the mRNA interferase activity of MazF-mx using MS2-0724 RNA as substrate (Figures 6A and 6B). When 50 ng of MazF-mx was preincubated with 200 ng of MrpC (a ratio of

Table 1. Cleavage Sites of MS2 ssRNA

Position[a]	Strength[b]	Cleavage[c]	Structure[d]
0724	+++	GA**GU!UGC**A	ss
0551	+/−	GU**GU!UGC**U	pd
0802	+/−	GA**GU!UUC**U	pd
0952	+/−	UG**GU!UG**UC	pd
0488	−	AC**GU UGC**G	ds
2175	NT	AA**GU UGC**A	ds
0602	+/−	CC**AU!UGC**G	pd
1106	+	AC**GU!AA**UA	ss
1241	+	AC**GU!AA**AG	ss
0449	−	CT**GU AA**GC	ds
0534	−	AG**GU AA**UU	ds
0639	−	GC**GU AA**UU	ds
1042	−	AG**GU AA**CA	ds
1127	+/−	UC**AU!AA**GC	ss
1109	+/−	UA**AU!AA**CG	pd
0213	+/−	AG**AU!AA**CU	ss
0929	−	AC**AU AA**AC	ds
1087	+	AU**GU!CA**GG	pd
0367	+/−	GG**GU!CA**UG	pd
0329	−	AA**GU CA**CA	ds
0524	−	AA**G**U **CA**CC	ds
0825	−	AC**GU CA**GG	ds
1124	+/−	CC**AU!CA**UA	ss
1289	+/−	UA**AU!CA**GG	ss
0342	−	AG**AU CA**AG	ds
0694	−	CG**A**U C**A**AG	ds
0842	−	AC**AU CA**AG	ss
Consensus		**GU!**	
		A	

[a] Position of U cleaved by MazF-mx at 3′-end in MS2 ssRNA.
[b] Strength of cleavage observed by the primer-extension analyses. NT, not tested.
[c] Cleavage site is indicated by ! and bold letter represents the residue sharing among cleavage sites.
[d] Predicted secondary structure in cleavage site; ss: single-stranded, ds: double-stranded, and pd: partially double-stranded.

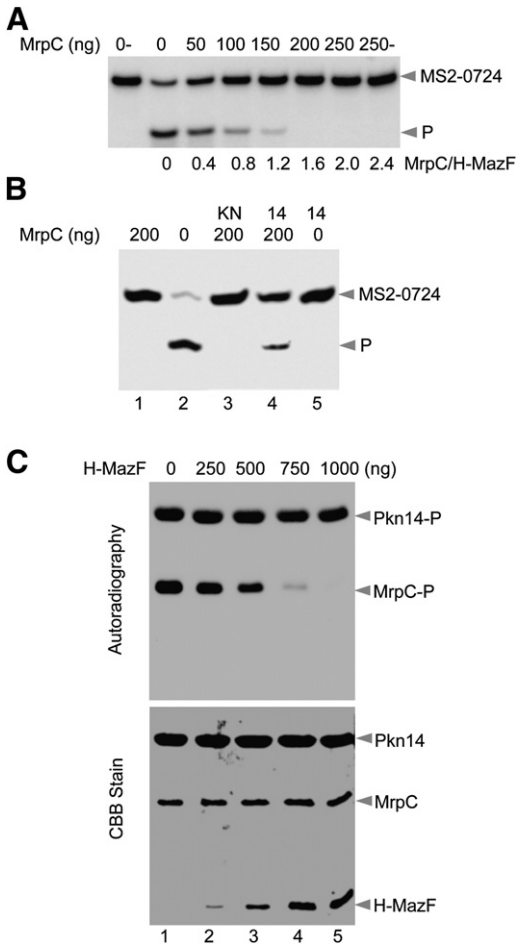

Figure 6. Effect of MrpC Phosphorylation by Pkn14 on Its Antitoxin Function for MazF-mx

0.01 pmole of MS2-0724 was incubated with H-MazF for 30 min at 30°C in 20 μl of MazF buffer. Reaction was stopped by addition of 12 μl sequencing loading buffer and heated at 95°C for 2 min and then placed on ice. The products were separated by 20% PAGE and subjected to autoradiography. MS2-0724 and the 5′-end-labeled cleavage product (P) are indicated by arrows.

(A) 50 ng of H-MazF was preincubated with MrpC for 10 min in MazF buffer. − indicates that no MazF was added.

(B) The effect of phosphorylation of MrpC by Pkn14 on its antitoxin function. H-MazF was incubated with Pkn14 (14) and Pkn14K48N (KN) (Nariya and Inouye, 2006) in the presence of ATP. After dialysis, samples were examined for their endoribonuclease activities.

(C) Phosphorylation of MrpC by Pkn14 in the MrpC-MazF complex. MrpC (2 μg) was preincubated with 0 (lane 1), 250 (lane 2), 500 (lane 3), 750 (lane 4), and 1000 ng (lane 5) in 10 μl of MazF buffer for 10 min at 30°C. Then, it was further incubated with Pkn14 (8 μg) in 20 μl of MazF buffer containing 10 mM $MgCl_2$ and 1 mM ATP/10 μCi [γ-^{32}P]ATP for 30 min. The reaction mixtures were subjected to 12.5% SDS-PAGE. The gel was transferred onto a PVDF membrane, followed by autoradiography and staining with CBB.

MrpC to MazF-mx of 1.6), MazF-mx-mediated cleavage of MS2-0724 was completely inhibited (Figure 6A and also compare lane 1 with lane 2 of Figure 6B). However, when MrpC was incubated with Pkn14 in the presence of 1 mM ATP, the inhibitory function of MrpC decreased (lane 4, Figure 6B), while the addition of Pkn14K48N had no effect on the MrpC activity (lane 3). Pkn14 by itself did not show any RNase activity (lane 5). Next we examined if MrpC in the complex with MazF-mx can be phopsphorylated by Pkn14. As shown in Figure 6C, free MrpC was effectively phosphorylated by Pkn14; however once MrpC formed the complex with MazF-mx, MrpC phosphorylation was significantly reduced. Together, these results support the notion that during vegetative growth, expression of the MrpC-MazF system is negatively regulated by the Pkn8-Pkn14 kinase cascade; MazF-mx has much higher affinity with the nonphosphorylated form of MrpC than with the phosphorylated form of MrpC so that nonphosphorylated MrpC is maintained at a very low level in the vegetative cells, which in turn suppresses the transcription of both *mrpC* anf *MazF* genes.

DISCUSSION

Together, our findings uncover that *M. xanthus* has a PCD cascade that is developmentally regulated and composed of an eukaryotic-like Ser/Thr protein kinase cascade (Pkn8-Pkn14), a developmental transcription factor/antitoxin (MrpC), and an mRNA interferase (MazF-mx). In this PCD cascade, the function of PCD-causing MazF-mx as interferase is inhibited by forming a complex with nonphosphorylated MrpC. The remaining free MrpC is phosphorylated by Pkn14 so that MrpC cannot activate transcription of the *mrpC* gene as well as the *mazF* gene. Therefore, the expression of the MrpC-MazF system is negatively regulated by a protein kinase cascade during vegetative growth. It is important to note that Pkn8 is essential for kinase activity for MrpC as the deletion of *pkn8* causes the same developmentally defective phenotype as the deletion of *pkn14*.

It has been demonstrated that during vegetative growth, MrpC is phosphorylated through the Pkn8-Pkn14 cascade, leading to repression of the transcription activator function of MrpC (Nariya and Inouye, 2006), as an activator for essential developmental genes, *mrpC* and *fruA*. Upon the onset of FB formation, the N-terminal 25 residue sequence of MrpC (where the phosphorylation site(s) is located) is processed presumably by LonD, a developmentally induced ATP-dependent protease, to form MrpC2, which in turn induces MrpC production as its function is no more affected by the Pkn8-Pkn14 cascade (Nariya and Inouye, 2006). MrpC2 is also likely to activate the *mazF-mx* gene expression, and subsequent cleavage of cellular mRNAs by MazF-mx may cause further devastating metabolic effects to the cells whose growth is already severely inhibited by nutrition deprivation. This may trigger autolysis by inducing a number of autolytic enzymes.

MrpC is a key regulator for activation of early developmental genes including *mazF-mx* (present results). During early and middle development, MrpC is expressed at a high level (Nariya and Inouye, 2006) that is likely able to neutralize MazF-mx toxicity, while it continues to upregulate the mazF-mx expression. Before sporulation is initiated, MrpC is proposed to be degraded by LonD and/or other unidentified cellular proteases (Nariya and Inouye, 2006), which then activate MazF-mx mRNA interferase function, resulting in developmental autolysis to provide nutrients for a minor population (20%) of cells, which are destined to form FB and subsequent myxospores. It should be noted that the extent of cell lysis during the fruiting body formation appears to be different from strain to strain of *M. xanthus*; and also different are the medium conditions used during the fruiting body formation, for example, little lysis was observed in the presence of *E. coli* cells as prey (Berleman and Kirby, 2007). It is tempting to speculate that the extent of PCD of *M. xanthus* during the fruiting body formation may be regulated by the availability of nutrition in the medium and probably also inside the cells.

How the 20% population is selected to survive avoiding autolysis remains an intriguing question. Since *M. xanthus* development does not uniformly occur, the seemingly altruistic autolysis may be a matter of timing, and the subpopulation in which the onset of the developmental program is delayed (maybe because of their position in the cell cycle at the time of nutritional deprivation) may be retriggered by transient release of nutrition from autolyzed cells to initiate the late developmental process. In this selected population, MazF-mx function has to be subdued by the mechanism yet to be determined. It is possible that the expression level of MrpC in this subpopulation may be relatively higher than that in the other lysing cells, effectively neutralizing MazF-mx toxicity to result in successful development. It is interesting to note that the *pkn8-pkn14* cascade deletion strain expresses a high level of MrpC even during vegetative growth, and that FB development progresses significantly faster than the parent strain (Nariya and Inouye, 2006) likely due to higher expression of MazF-mx. It also remains to be elucidated if MazF-mx can trigger PCD through the cleavage of a specific mRNA(s) or rather does this by inflicting general damage to the cells as suggested in the case of *E. coli* MazF (Engelberg-Kulka et al., 2005). In this regard, it is also interesting to note that MazF induction in mammalian cells induces BAK-dependent PCD (Shimazu et al., 2007).

The prevailing TA systems in bacteria appear to have multiple functions in bacterial physiology, although there are conflicting views on the roles of the TA systems in PCD in *E. coli*; a recent report demonstrated that MazF mediates cell death (Kolodkin-Gal et al., 2007), while another group disputed that it does not cause PCD (Tsilibaris et al., 2007). Our results demonstrate that solitary MazF has a distinct mission from those toxins encoded by an operon together with their cognate antitoxin, as it functions only for PCD (rather than cell growth arrest) in a sophisticated PCD cascade associating with Ser/Thr protein kinases, which is reminiscent of the eukaryotic PCD cascade.

EXPERIMENTAL PROCEDURES

Bacteria, Growth Conditions, Plasmid, and DNA Manipulation

M. xanthus FB (DZF1) (Morrison and Zusman, 1979) and its derivatives were cultured in CYE medium at 30°C (Campos et al., 1978) supplemented with 80 μg/ml kanamycin or 250 μg/ml streptomycin when necessary. To initiate fruiting body development, *M. xanthus* cells were spotted on CF (Hagen et al., 1978) and TM agar (Nariya and Inouye, 2003) plates and spore yields were measured as described previously (Inouye et al., 1979). Autolysis during development was measured by counting cell numbers (Nariya and Inouye, 2003). Cell viability was examined by measuring colony formation units (CFU) by plating cells on CYE plates. *E. coli* DH5α (Hanahan, 1983) was used as the recipient strain for transformation and grown in LB medium (Miller, 1972) supplemented with 50 μg/ml kanamycin, 100 μg/ml ampicillin, or 25 μg/ml streptomycin. *E. coli* BL21 (DE3) was used for the expression of *mazF-mx* under the control of a T7 promoter in a T7 vector (Studier et al., 1990). The proteins were induced by the addition of 1 mM IPTG at 100 Klett (equivalent to 5×10^8 cells/ml) in M9 medium (Maniatis et al., 1989) supplemented with 100 μg/ml ampicillin. pUC19 (Yanisch-Perron et al., 1985) was used to clone chromosomal DNA fragments. DNA sequences were determined by an ABI Genetic Analyzer 310 using the methods provided by the company and double-stranded plasmid DNA as templates. *M. xanthus* genomic DNA was used as template for PCR amplification. PCR-amplified regions were confirmed by DNA sequencing. Other DNA manipulations were carried out by the methods described previously (Munoz-Dorado et al., 1991).

Construction of a *mazF-mx* In-Frame Deletion Strain, Δ*mazF*, and a *mazF-mx-lacZ* Fusion Strain

A method developed based on the cell toxicity by *galK* (galactokinase gene) (Ueki et al., 1996) was used for construction of an in-frame deletion of MazF-mx between Pro24 and Ser100. Since the genomic database for *M. xanthus* (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org=gmx) shows that *M. xanthus* does not contain *galK* and *galT* (galactose-1-phosphate

uridylyltransferase gene), D-(+)-galactose can be used in this system in place of 2-deoxygalactose. Two PCR fragments (MazF-N, 577 bp and MazF-C, 566 bp) were amplified using the *M. xanthus* chromosomal DNA as template by the following primers: one fragment with MazF-N5 (AAAGAATTCAAGC TTCGAACCAGCGCAGGCGGTTGTAGAGGCAT) and MazF-N3 (AAAGGATC CAAA**GTCGAC**CGGGCCTCGTGAGTCGTCGGGCTCCA), and the other fragment with MazF-C5 (AAAGAATTCAAGCTT**GTCGAC**GCGCGGGTGGAACA GATTCTTGCC) and MazF-C3 (AAAGGATCCTCAAGACGAGCCCGCCAGC GAAGAGCACT). These fragments were cloned into pKO1Km^R (Ueki et al., 1996) at EcoRI and BamHI sites resulting in plasmids, pMazF-N and pMazF-C, respectively. The SalI-BamHI fragment from pC-MazF was inserted into pMazF-N at SalI-BamHI, resulting in pMazF-IF, which has an in-frame fusion between Val23 (GTC) and Asp101 (GAC). pMazF-IF was electroporated into DZF1 cells for single crossing-over recombination (1st recombination) to screen kanamycin-resistant cells on CYE plates containing 80 μg/ml kanamycin. Kanamycin-resistant colonies were then subjected to colony-directed PCR to determine the sites of integration, using the following primers: for upstream integration (N-cross), MazF-5 (GTGGGCGCGAAGTGCGCAGCCGTG TCT) and Km-1 (CTGGCTTTCTACGTGTTCCGCTTCCTTTAGC) in pKO1Km^R, and for downstream integration (C-cross), MazF-5 and MazF-IC (TCGTCG TCGTGTCGCAGGTGTCCTCGGT). N- and C-cross strains identified above were individually cultured in CYE medium to 100 Klett, and then serially diluted cultures with CYE medium were plated on CYE agar plates containing 10 mg/ml D-(+)-galactose (Sigma). Kanamycin-sensitive and galactose-resistant colonies that resulted from the second recombination looping out the plasmid-derived region were from either the original wild-type, DZF1, or in-frame deletion strains (*ΔmazF*). The Δ*mazF* mutation was identified by colony-directed PCR using two sets of primers: one with MazF-5 and MazF-I (GAGT GATTGAAGACGTCGTCCTGAACCACCA) and the other with MazF-5 and MazF-C3. Since the phenotype during vegetative growth and development of both Δ*mazF* strains obtained from both N- and C-cross was identical, they were used as Δ*mazF*.

The *lacZ*-fusion strain with the *mazF-mx* promoter region was constructed by insetting MazF-N fragment (−344 to +233) digested with HindIII and BamHI into pZK (Nariya and Inouye, 2005b), resulting in pZK-*mazF*^{p}. β-galactosidase assays were carried out as described previously (Kroos et al., 1986; Nariya and Inouye, 2005b).

LIVE/DEAD Staining

5 × 10^8 cells (vegetative cells and developmental cells spotted on CF plate) washed twice with TM buffer were stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen; L7012) according to the manufacturer's protocol. Stained cells were concentrated to 10^9 cells/ml in TM buffer and the pictures were taken using a fluorescence microscope (OLYMPUS BX-FLA).

Primer-Extension Analysis

Total RNA was isolated by the hot-phenol method from DZF1, Δ*mrpC*, and Δ*pkn14* cells grown in CYE medium harvested at the early-log (12 hr/50 Klett), mid-log (16.5 hr/100 Klett), late-log (24 hr/200 Klett), early-stationary (36 hr/350 Klett), mid-stationary (48 hr/350 Klett), and late-stationary (60 hr/280 Klett) phases (Nariya and Inouye, 2005b). At the early-stationary phase, cells were spotted on TM agar plates to initiate fruiting body development, and developmental cells were collected at 0, 6, 12, and 24 hr as described previously (Nariya and Inouye, 2005b). Primer extension was carried out using primer MazF-AS as described previously (Nariya and Inouye, 2003). The extended products were analyzed on a 6% polyacrylamide gel containing 8 M urea with a sequencing ladder made with the same primer using pMazF-N as template.

Construction of *M. xanthus* Expression Vector, pKSAT

Since the kanamycin resistance gene (*km^r*) from Tn5 is generally used as a drug marker in *M. xanthus* and known to be constitutively expressed during both vegetative growth and development, its promoter region (159 bp) was amplified by PCR with primers Km-P5 (AAAGGTACCACAGCAAGCGAACCG GAATTGCCA) and Km-P3 (AAACATATGAAACGATCCTCATCCTGTCTC) using pUC7Km(P-) as template (Norioka et al., 1995). The resulting DNA fragment was cloned into pBluescript II SK(-) (Stratagene) between KpnI and NdeI sites, resulting in pKA. The 1.9 kbp NdeI-HincII fragment containing *strA-strB* genes from *Salmonella typhimurium* plasmid R64 (Komano et al., 2000) was then inserted between two SspI sites in pKA, resulting in pKS. For *attB*/*attP* recombination in *M. xanthus*, the 2.9 kbp SmaI fragment containing *intP-attP* from Myxophage Mx8 (Tojo et al., 1996) was inserted between two DraI sites, resulting in pKSAT. In this plasmid, the transcription directions of both *strA-strB* and *intP-attP* were selected to be the same as that of the *km^r* promoter. pKSAT contains NdeI and BamHI sites for cloning genes for expression.

Yeast Two-Hybrid Screen for Identification of the Antitoxin for MazF-mx

The 0.4 kb NdeI-BamHI fragment from *mazF-mx* was amplified by PCR using primers, MazF-N (AAACATATGCCCCCCGAGCGAATCAACCGCGGTGA) and MazF-C (AAAGGATCCTCACGGCCTCGCGAAGAACGACACCTGCT), and cloned into pGBD-NdeI for bait and pGAD-NdeI for target to perform a yeast two-hybrid screen (Nariya and Inouye, 2005b). The yeast strain PJ69-4A was used for the yeast two-hybrid screen (James et al., 1996) and the *M. xanthus* genomic DNA library used is described previously (Nariya and Inouye, 2005b). Interaction between MazF-mx and MrpC in the yeast two-hybrid screen was examined by a quantitative β-galactosidase activity assay (Nariya and Inouye, 2005b).

Expression and Purification of MazF-mx

The *mazF-mx* fragment was cloned into pET-11a and pET-16b(+) (Novagene) resulting in pET-MazF or pET-H-MazF, respectively. Both nontagged MazF-mx and N-terminal histidine-tagged MazF-mx (H-MazF) induced in *E. coli* BL21 (DE3) by IPTG for 3 hr were soluble. H-MazF was purified using Ni-NTA SUPER FLOW resin (QIAGEN) as described before (Nariya and Inouye, 2005a). The eluted fraction from the resin was then dialyzed against 50 mM Tris-HCl, pH 8.0, containing 20% (w/v) glycerol, followed by passing through HiTrap SP and Q columns (GE). H-MazF was recovered from the flow-through pool by the resin. The eluted fraction was dialyzed against MazF buffer (25 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, 5%[w/v] glycerol, and 0.5 mM DTT], and purified H-MazF (0.5 mg/ml) was stored at −80°C. Gel-filtration analysis using purified H-MazF (200 μl) was performed as described previously (Nariya and Inouye, 2005a). H-MazF (15.9 kD on SDS-PAGE) was eluted at the position of ~30 kD (dimer).

Interaction of MazF-mx with MrpC

A pull-down assay was carried out as previously described (Nariya and Inouye, 2005b). Five hundred microliters of crude soluble fraction (S) from *E. coli* (2000 Klett/ml) expressing nontagged MazF-mx was incubated with (+) or without (−) 25 μg of purified N-terminal histidine-tagged MrpC (Nariya and Inouye, 2005a). The complex was recovered by 10 μl of the Ni-NTA resin. The complex thus formed was analyzed by SDS-PAGE. Immunoprecipitation was carried out using the soluble fraction (500 μl) from the mid-log phase cell suspension (2000 Klett/ml MazF buffer with 5 mM EDTA and 2× concentration of protease inhibitor cocktail [Roche]) and anti-MrpC rabbit IgG (50 μl), as described previously (Nariya and Inouye, 2005a, 2006). Immumoprecipitates were then dissolved in 50 μl of 1×SAB containing 250 mM NaCl, and then 10 μl of samples were subjected onto a 15% SDS-PAGE, followed by western blot analysis using anti-MrpC IgG and monoclonal anti-HA mouse IgG (Nariya and Inouye, 2005b) or anti-MazF-mx mouse serum raised by purified H-MazF (PRF&L).

Expression of MazF-mx in *M. xanthus*

Hemagglutinin epitope (HA)-tagged *mazF-mx* was amplified by PCR using primers MazF-HA (AAACATATGGGGTACCCCTACGACGTGCCCGACTACG CCATGCCCCCCGAGCGAATCAACCGCGGTGA) and MazF-C. The HA-tagged and nontagged *mazF-mx* genes were then cloned into pKSAT at NdeI and BamHI sites resulting in plasmids pKSAT-MazF and pKSAT-HA-MazF, respectively. They were integrated into the chromosome of Δ*mazF* and Δ*mrpC* by site-specific (*attB*/*attP*) recombination (Nariya and Inouye, 2003) resulting in strains pKSAT-MazF/Δ*mazF* and pKSAT-HA-MazF/Δ*mrpC,* respectively. pKSAT was also integrated into Δ*mazF* and Δ*mrpC* strains, resulting in strains pKSAT/Δ*mazF* and pKSAT/Δ*mrpC*, respectively. pKSAT-Pkn14 and pKSAT–Pkn14KN were also constructed using the corresponding NdeI and BamHI

fragments (Nariya and Inouye, 2006), which were then integrated into the chromosome of DZF1.

Expression of MazF-mx in Δ*mrpC* (10^8 cells) carrying pKSAT-HA-MazF during vegetative growth was detected by western blot using HA antibody (Nariya and Inouye, 2005b).

Gel-Shift Assay

The promoter region of *mazF-mx* (P*mazF*: −73 to +166) was amplified by PCR using primers MazF-N5 and MazF-N3. The product was purified by agarose gel electrophoresis using the QIAquick Gel Extraction Kit (QIAGEN). Purified P*mazF* was then labeled at the 5′ end with [γ-^{32}P]ATP by T4 kinase (Invitrogen), followed by further purification using the QIAquick PCR purification Kit (QIAGEN). The gel-shift assay was carried out using purified MrpC and labeled P*mazF* (10 fmoles) as described previously (Nariya and Inouye, 2006). MrpC was incubated with H-MazF in 5 μl of MazF buffer for 10 min at 30°C and subjected to the gel-shift assay.

mRNA Interferase Activity of MazF-mx

M. xanthus total RNA isolated from mid-log cells was treated with 1 mM ATP and T4 kinase on ice for 60 min to mask all the free 5′-ends and purified on a QIAGEN column using PB and PE buffers (QIAGEN). Purified RNA (0.1 μg) was digested with H-MazF in 20 μl of MazF buffer for 30 min at 30°C. Products were then labeled with [γ-^{32}P]ATP by T4 kinase. Denatured products in urea were separated on 1.2% TBE native agarose gel (Liu and Chou, 1990). The gel was stained with ethidium bromide (EtBr) and then dried with a gel drier. The dried gel was subjected to autoradiography.

MS2 ssRNA (0.8 μg; 3569 bases; Roche) was digested by H-MazF in 20 μl of MazF buffer at 30°C as indicated. H-MazF was preincubated with MrpC for 10 min and then further incubated with MS2 ssRNA for 30 min. MrpC (2.5 μg) was incubated with 10 μg of Pkn14 (14) or autokinase-defect mutant, Pkn14K48N (KN) (Nariya and Inouye, 2006), in 50 μl of P buffer with 1 mM ATP at 30°C for 4 hr, followed by dialysis against MazF buffer containing 200 mM NaCl at 4°C. Four microliters (200 ng MrpC) of dialysates were preincubated with H-MazF (50 ng) in 20 μl of MazF buffer for 10 min at 30°C. To this solution, 0.01 pmole of 5′-end γ-^{32}P-labeled MS2-0724 (a 14 base synthetic RNA substrate; see the text) was added and the mixture was incubated for 30 min at 30°C. For control, MS2-0724 was incubated with only Pkn14. Reactions were stopped by addition of 12 μl of sequencing loading buffer (Stop Solution; Roche) and heated at 95°C for 2 min and then placed on ice. The product was separated by a 20% TBE-PAGE and the gel was subjected to autoradiography.

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