Journal of Cellular Biochemistry 51:29–33 (1993)

Eukaryotic-Like Protein Serine/Threonine Kinases in *Myxococcus xanthus*, a Developmental Bacterium Exhibiting Social Behavior

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Abstract *Myxococcus xanthus*, a gram-negative bacterium exhibits a spectacular life cycle and social behavior. Its developmental cycle and multicellular morphogenesis resemble those of eukaryotic slime molds such as *Dictyostelium discoideum*. On the basis of this resemblance, we explored the existence of eukaryotic-like protein serine/threonine kinases which are known to play important roles in signal transduction during development of *D. discoideum*. It was indeed found that *M. xanthus* contains a large family of protein serine/threonine kinases related to the eukaryotic enzymes. This is the first unambiguous demonstration of eukaryotic-like protein serine/threonine kinases in the prokaryotes.

Key words: protein kinases, myxobacteria,

THE DEVELOPMENTAL CYCLE OF *Myxococcus xanthus*

Myxococcus xanthus, a myxobacterium, has attracted the attention of many scientists during the last two decades because of its spectacular life cycle and social behavior. *M. xanthus* is a soil-dwelling Gram-negative bacterium capable of degrading a wide variety of complex macromolecules by means of extracellular hydrolytic machinery [Rosenberg and Varon, 1984]. Cells always move as a "swarm" gliding on solid surfaces. This large multicellular community thus effectively preys on other microorganisms and macromolecules by utilizing extracellular degradative enzymes. This social behavior becomes more evident upon nutrient depletion; cells start moving toward aggregation centers where they pile up on top of each other to build macroscopic mounds known as fruiting bodies. Inside the fruiting bodies the vegetative rod-shaped cells differentiate into spherical dormant myxospores which become resistant to severe environmental conditions such as heat and desiccation. Myxospores germinate to resume vegetative growth when nutritional conditions are favorable again (see Fig. 1 and Kaiser, 1986; Shimkets, 1990; Dworkin, 1991).

Received May 9, 1992; accepted May 9, 1992.

The developmental cycle of *M. xanthus* responds to a very specific program in which many different types of genes have been found to be spatially (rod-shaped cells or myxospores) and temporally regulated [Inouye et al., 1979; Zusman, 1984; Kroos et al., 1986]. The expression of some late genes in development is dependent on the expression of earlier developmental genes [Kroos and Kaiser, 1987]. Recently, several genes encoding sigma factors have been reported in *M. xanthus* [Inouye, 1990; Apelian and Inouye, 1990; submitted], and two of them have been shown to be expressed at different times during development: the *sigC* gene is expressed at an early stage of development in the rod-shaped cells [Apelian and Inouye, submitted], while *sigB* is expressed late in the development and only inside the myxospores [Apelian and Inouye, 1990]. These sigma factors are considered to play important roles in regulating gene expression during development. In fact, a null mutation of *sigB* has been found to be defective in the expression of *ops*, a gene that is also expressed exclusively in the myxospores [Feintze et al., 1985].

The developmental cycle and multicellular morphogenesis of *M. xanthus* (Fig. 1) resemble those of eukaryotic slime molds such as *Dictyostelium discoideum* [Kaiser, 1986; Kessin, 1988;

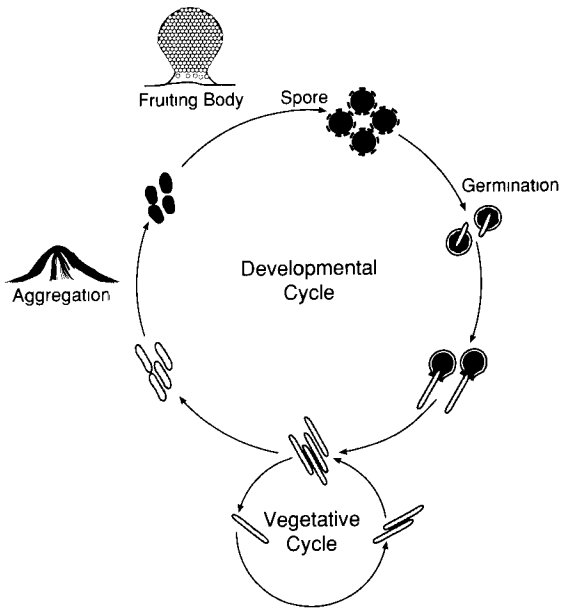

Fig. 1. Life cycle of myxobacteria. When nutrients are depleted, vegetative cells aggregate to form fruiting bodies on a solid surface, and the rod-shaped cells convert to round or oval myxospores which become resistant to many environmental stresses such as desiccation and high temperature.

Derreotes, 1989]. *D. discoideum* is known to possess a signal transduction system consisting of a receptor, a G protein, an effector, and a multigene family of protein serine/threonine kinases which are developmentally regulated and are considered to play important roles in development [Klein et al., 1988; Kumagai et al., 1989; Haribabu and Dottin, 1991]. These protein kinases belong to a large family of protein serine/threonine kinases widely prevailing in eukaryotic cells [Edelman et al., 1987]. However, in the prokaryotes, various signal transduction systems required for adaptation to numerous environmental signals are known to consist of protein histidine kinases [Stock et al., 1989], and signal transduction through protein serine/threonine or tyrosine kinases has been considered for a long time to be specific to eukaryotic cells [Edelman et al., 1987; Hunter and Cooper, 1985].

This dogma was recently challenged, and it was found that at least *M. xanthus* contains a large family of protein serine/threonine kinases related to the eukaryotic enzymes [Zhang et al., 1992]. On the basis of the resemblance of the *M. xanthus* life cycle to that of *D. discoideum*, it was speculated that the signal transduction systems similar to those in eukaryotic cells might play an important role during *M. xanthus* development. In this review we describe these eukaryotic-like protein serine/threonine kinases found in *M. xanthus*.

IDENTIFICATION OF PROTEIN SERINE/THREONINE KINASES IN *M. xanthus*

The catalytic domain of eukaryotic protein kinases consists of 11 subdomains some of which contain highly conserved consensus amino acid sequences [Hanks et al., 1988]. The oligonucleotides designed on the basis of the consensus sequences in the subdomains VI and VIII of protein serine/threonine kinases were used as primers in polymerase chain reaction (PCR) with the *M. xanthus* chromosomal DNA as a template [Munoz-Durado et al., 1991]. When the PCR reaction products were analyzed on a gel, it was found that a major band migrated at a position of approximately 175bp. After cloning the band into a plasmid, its DNA sequence was determined. On the basis of the amino acid sequence deduced from the DNA sequence, it was found that there were three different sequences in all, of which subdomain VII containing the highly conserved amino acid sequence, Asp-Phe-Gly, was flanked with the amino acid sequences of subdomains VI and VIII in the same reading frame. This result suggested that *M. xanthus* contains at least three eukaryotic-like protein kinases.

CHARACTERIZATION OF A PROTEIN SERINE/THREONINE KINASE, PKN1, IN *M. xanthus*

The first *M. xanthus* protein kinase gene, *pkn1*, was isolated using PCRPK1, one of the PCR products, as a probe, and its DNA sequence was subsequently determined. The amino acid sequence deduced from the DNA sequence consists of 693 residues, and its amino-terminal half contains all the consensus sequences found in the catalytic domain of eukaryotic protein serine/threonine kinases. Eukaryotic protein serine/threonine kinases are classified into four types depending upon their catalytic and regulatory domain structures [Edelman et al., 1987]. Ca^{++}/calmodulin-dependent protein kinaseII (CAM-kinaseII) is known to have a catalytic domain at the amino-terminal region and a regulatory domain at the carboxy-terminal region. When the amino acid sequence from residue 57 to 264 in Pkn1 is compared with the catalytic domain of rat CAM-kinaseII as shown in Figure 2 [Tobimatsu and Fujisawa, 1989], the identity

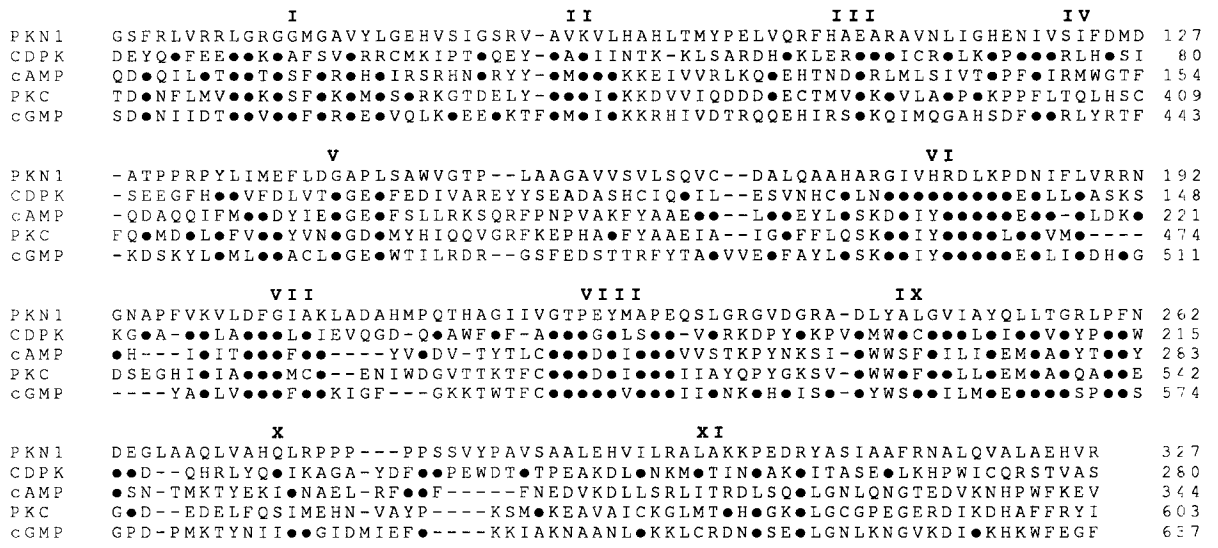

Fig. 2. Sequence alignment in the region between domains I and XI of Pkn1 and other eukaryotic proteins kinases. CDPK, rat Ca^{2+}/calmodulin-dependent protein kinasesII [Tobimatsu and Fujisawa, 1989]; cAMP, yeast cAMP-dependent protein kinase [Toda et al., 1987]; PKC, rabbit protein kinase Cα [Ohno et al., 1987]; and cGMP, human cGMP-dependent protein kinase [Sandberg et al., 1989]. Amino acid residues identical to Pkn1 are represented by dots, and bars represent gaps. The assignment of subdomains was defined by Hanks and Quinn [1991].

between them is 31% which is higher than those with other protein kinases (27% with rabbit protein kinase Cα [Ohno et al., 1987], 28% with yeast cAMP-dependent protein kinase [Toda et al., 1987], and 30% with human cGMP-dependent protein kinase [Sandberg et al., 1989]). The carboxy-terminal domain of CAM-kinaseII has been shown to be involved in regulation, subunit assembly, and subcellular localization [Soderling, 1990]. In addition, it has been shown that this kinase can recognize the sequence RXXS/T and phosphorylate the serine or threonine residue within the kinase sequence [Golbran et al., 1988].

Phosphorylated amino acid residues in Pkn1 were determined by expressing *pkn1* using the T7 RNA polymerase system [Munoz-Dorado et al., 1991]. Serine and threonine residues were found to be equally phosphorylated but not tyrosine residues. This autophosphorylation reaction was blocked when the lysine residue at position 88 in Pkn1 was replaced with an asparagine residue. The sequence, RXXS/T, recognized as the phosphorylation site by CAM-kinaseII, exists at three locations in Pkn1: RQRS (residues 517–520), RLRT (540–543), and RARS (689–692) in Pkn1 [Munoz-Dorado et al., 1991]. These similarities in sequence and the domain structure between *pkn1* and CAM-kinaseII are quite interesting since a major Ca^{2+}-binding protein (protein S; Inouye et al., 1979; Teintze et al., 1988) is induced during myxospore formation.

FUNCTION OF PKN1 DURING THE DEVELOPMENT OF *M. xanthus*

By analyzing β-galactosidase activity of the *M. xanthus* cells harboring a *pkn1-lacZ* fusion gene it was found that the *pkn1* gene was expressed only during development, but not during vegetative growth. When *M. xanthus* cells carrying the *pkn1-lacZ* gene [Munoz-Dorado et al., 1991] were placed on a low nutrient agar (CF agar) plate with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as a *lacZ* indicator at 30°C, the blue color induction occurred after approximately 30 h incubation, when well-defined fruiting bodies were formed. Further characterization revealed that *pkn1* expression was induced before sporulation and continued inside myxospores.

Δ*pkn1* deletion mutants (Δ*pkn1a* and b) were constructed to investigate the function of *pkn1* during development. These mutant cells grew normally in rich media, but they were defective in fruiting body formation. Surprisingly, Δ*pkn1* strains started aggregation approximately 4–8 h earlier than the wild-type strain. Its fruiting bodies were smaller and not compact when compared to those of the wild-type strain. The spore formation of the Δ*pkn1* strains also started earlier by the same timing of its fruiting body

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Consensus       D K  N                        D G                                    PE
PKN 1       IVHRDLKPDNIFLVRRNG-----NAPFVKVLDFGIAKLADAHMPQTH------AGIIVGTPEYMAPEQS
PKN 2       ●I●●●●●●E●V●ISKSARG------EQARL●●●●●●R●VEPDAASSVSQ----I●VVL●●●●●LS●●●A
PKN 3       VI●●●●●●E●●MVEP●RNEP-----D●●●●●●●●●●●IT●STDDGPALTR---●●FVC●●●●●●S●●●A
PKN 5       L●●●●●●●S●●MVDDDR          Q●RLM●●●L●●FLADDAAI●E------●●KL●●●YR●●●●●●I
PKN 6       LI●●●VS●●●●LVS●QG---------A●●●V●●●●●●V●GQGHRTL-------T●VVK●KVA●●P●●●L
PKN 11      L●●●●VT●H●VL●SFDG---------A●●LT●●●●●●AGNKLTQ---------P●VLK●KFA●●S●●●A
PKN 13      VI●●●VAKE●●MVTYEG---------VT●L●●●●●●●SLARADRTA-------V●MVK●●SG●●S●●●I
PKN 15      VI●●●●●●E●VMVREDG---------VL●LM●●●●●R●M●IEERM●V------T●TL●●S●AH●●●●II

Subdomain       VIb                          VII                                VIII
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Fig. 3. Sequence comparisons among *M. xanthus* protein kinases. The regions for subdomains VIb, VII, and VIII are shown for Pkn1 [Munoz-Dorado et al., 1991], 2, 3, 5, 6, 11, 13, and 15. Identical residues to Pkn1 are represented by solid circles. Highly conserved residues in eukaryotic protein serine/threonine kinases [Hanks and Quinn, 1991] are shown on the top as consensus residues.

formation and reached a plateau much sooner than that of the wild-type strain. As a result, the spore yield of the Δ*pkn1* strains was approximately 35% of that of the wild-type strain. These results indicate that Pkn1 is required for the normal development of *M. xanthus,* and that it probably involves sensing the signal for determination of the exact timing for initiation of fruiting body formation.

MULTIGENE FAMILY OF PROTEIN SERINE/THRONINE KINASES IN *M. xanthus*

During the identification of the *pkn1* gene using PCRPK1 as a probe for Southern blot analysis of the *XhoI* digest of the chromosomal DNA, another band was found to hybridize with the probe in addition to the *pkn1* band [Munoz-Dorado et al., 1991]. When hybridization was performed under a less stringent condition using the same probe [Zhang et al., 1992], 12 additional bands were detected with different intensities.

Furthermore, during the course of the experiments identifying PCRPK1 from the PCR products described above, two other DNA fragments consisting of 173 and 176 bp, respectively, were obtained, and their DNA sequences revealed that they also represented regions encompassing well-conserved domains VI, VII, and VIII for new *pkn* genes. It was found that these three PCR products (PCRPK1, PCRPK2, and PCRPK3) shared the identical sequence consisting of 16 bases, $^{5'}$TGGACTTCGGCATGCG$^{3'}$ within subdomain VII. When this sequence was used as a probe, at least 23 bands were detected in the *Xho*I digest of the chromosomal DNA out of which 12 hybridized to PCRPK1 [Zhang et al., 1992]. The DNA fragments corresponding to putative *pkn* genes were identified in an *M. xanthus* genomic λ library using PCRPK1 as a probe. Partial DNA sequences encompassing the catalytic domain of some of these putative protein kinases have been determined. Amino acid sequences of the region encompassing domains VI, VII, and VIII were deduced from the DNA sequences of seven independent clones and compared with that of Pkn1. As shown in Figure 3, they indeed represent the well-conserved kinase sequences, thus indicating that all of them were derived from eight independent protein serine/threonine kinases.

Using DNA fragments of *pkn2, 3, 5, 6, 11, 13,* and *15* as probes one could further characterize the relatedness of all the bands detected in Southern blot hybridization. From these analyses, there appears to be at least 26 *pkn* genes in *M. xanthus* [Zhang et al., 1992].

REMARKS

The finding of a large family of eukaryotic-like protein serine/threonine kinases in *M. xanthus* narrows the gap between the prokaryotes and the eukaryotes raising a number of interesting questions as to the origin of protein serine/threonine kinases and their roles in various cellular functions. It is interesting to note that the same PCR approach has so far been unsuccessful for *Escherichia coli,* while the *pkn1* gene probe is able to hybridize with several DNA fragments from the chromosomal DNA of *Stigmatella aurantiaca,* another developmental myxobacterium (unpublished results). These results suggest that eukaryotic-like protein serine/threonine kinases may be found only in bacteria with a developmental life cycle. The finding that Pkn1 plays a role in fruiting body formation of *M. xanthus* supports this notion. However, in light of the fact that *M. xanthus* contains a large number of protein serine/threonine kinases, one cannot rule out the possibility that some of the

kinases may be associated with cellular functions during vegetative growth.

Although studies on bacterial protein serine/threonine kinases have recently begun, it will not be too long before we will learn the functions of these protein kinases. The excellent genetic systems available in *M. xanthus,* and the fact that at least some of the kinases will only be required for development but not for vegetative growth are expected to make functional and biochemical analysis of these kinases much easier than those in the eukaryotic cells.

Listed below are some questions which still need to be answered:

1) Does *M. xanthus* have signal transduction cascades with protein serine/threonine kinases involved?
2) Does *M. xanthus* have protein tyrosine kinases which may function as initial sensors for external signals?
3) Does *M. xanthus* have a signal transduction system consisting of a receptor, a G protein, an effector, and a protein kinase as found in eukaryotes?
4) Does *M. xanthus* have protein phosphatases for the target proteins of protein kinases? In this regard it is interesting to note that alkaline, neutral, and acid phosphatase activities are induced during the development of *M. xanthus* [Weinberg and Zusman, 1990].

ACKNOWLEDGMENTS

The authors wish to thank Mr. Jack McNulty for the critical reading of this manuscript. The work described in this paper was supported by a grant from the National Institutes of General Medical Sciences (GM44012 to M.I.).

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