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NOTES

Identification of a Putative Eukaryotic-Like Protein Kinase Family in the Developmental Bacterium *Myxococcus xanthus*

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Myxococcus xanthus **is a gram-negative bacterium which, upon starvation, undergoes a spectacular developmental cycle culminating in the formation of spore-filled fruiting bodies. We recently characterized a protein serine-threonine kinase (Pkn1) that is required for normal development (J. Munoz-Dorado, S. Inouye, and M. Inouye, Cell 67:995–1006, 1991).** ***pkn1*** **was cloned by polymerase chain reaction amplification with primers designed from conserved sequences in eukaryotic protein kinases. In this study, a fragment of the** ***pkn1*** **gene and an oligonucleotide corresponding to another highly conserved region were employed as probes for Southern blot analyses, which indicated that there are at least 26 putative kinase genes in** ***M. xanthus*****. Most of the putative kinase genes were cloned, and complete or partial sequencing of eight clones revealed that they indeed contained highly conserved sequences present in eukaryotic kinases. These results suggest that complex kinase cascades similar to those described for eukaryotes might be involved in regulation of the** ***M. xanthus*** **life cycle.**

Myxococcus xanthus is a gram-negative soil bacterium which is distinguished from other bacteria because of its remarkable social behaviors and adaptive responses. *M. xanthus* is capable of gliding motility, intercellular signalling, cell-cell interaction, and the formation of multicellular fruiting bodies containing myxospores (for a review, see reference 12). The developmental cycle and multicellular social morphogenesis of this bacterium resemble those of eukaryotic slime molds such as *Dictyostelium discoideum*. *D. discoideum* is a typical eukaryote in the sense that transmembrane signalling is accomplished via receptors, G proteins, and protein tyrosine or serine-threonine kinases. Components of such signalling pathways are required for developmental morphogenesis (for reviews, see references 1 and 4). Despite the gross morphological similarities between *M. xanthus* and *D. discoideum*, it is not clear whether similar gene products would be required for signal transduction.

Many membrane signalling pathways in bacteria employ histidine kinases whose amino acid sequences bear no similarities to protein serine-threonine or tyrosine kinases employed by eukaryotes (for a review, see reference 14). However, recently, we have found that *M. xanthus* contains a protein serine-threonine kinase with an amino acid sequence that is very similar to that of eukaryotic protein serine-threonine kinases (9). This protein kinase (Pkn1) undergoes autophosphorylation at serine and threonine residues, and deletion of the *pkn1* gene resulted in premature differentiation, with a poor yield of myxospores. It was proposed that Pkn1 plays an important role in the proper initiation of differentiation under a nutrient-deficient growth condition. In this report, we further examine whether *M. xanthus* harbors a eukaryotic-like kinase family. It was found that *M. xanthus* contains at least 26 different putative kinase genes.

Eukaryotic cells are known to contain a large family of protein kinases (for a review, see reference 5). The catalytic domains of the eukaryotic protein serine-threonine kinases consist of 11 subdomains, some of which contain highly conserved amino acid residues (3). To determine how many genes for protein serine-threonine kinases exist in *M. xanthus*, Southern blot analysis was carried out by employing the 170-bp *Bam*HI fragment of PCRPK1 (9) as a probe (encompassing subdomains VIb, VII, and VIII). Figure 1A shows the results obtained when the *M. xanthus* chromosomal DNA was digested with three different restriction enzymes and hybridized with PCRPK1 as a probe under the condition that allowed one to detect DNA sequences of at least 70% sequence homology (see Fig. 1). Thirteen bands were detected in various intensities on digestion with *Xho*I (lane 1), among which band 15 (4.9 kb) corresponds to *pkn1*.

During the polymerase chain reaction analysis employed to isolate the *pkn1* gene, two other DNA fragments were also cloned from the same polymerase chain reaction products. These were designated PCRPK2 and PCRPK3, consisting of 173 and 176 bp, respectively. The DNA sequencing of these fragments revealed that they also represent regions encompassing subdomains VIb, VII, and VIII of new *pkn* genes (data not shown). PCRPK1, PCRPK2, and PCRPK3 all were found to contain the identical 16-bp sequence within subdomain VII, shown in Fig. 1C (boxed sequence). When this sequence was used as a probe (oligo3019), at least 23 bands were detected in the *Xho*I digest of the chromosomal DNA (Fig. 1B, lane 1). Southern blot hybridization was performed at 42°C in a mixture consisting of 5× Denhardt's solution, 6× NET (1× NET is 150 mM NaCl–15 mM Tris-HCl [pH 7.5]–1 mM EDTA), 10% dextran sulfate, and 0.5% sodium dodecyl sulfate. The filter was washed until λ DNA fragments, used as size markers, became negative. These bands

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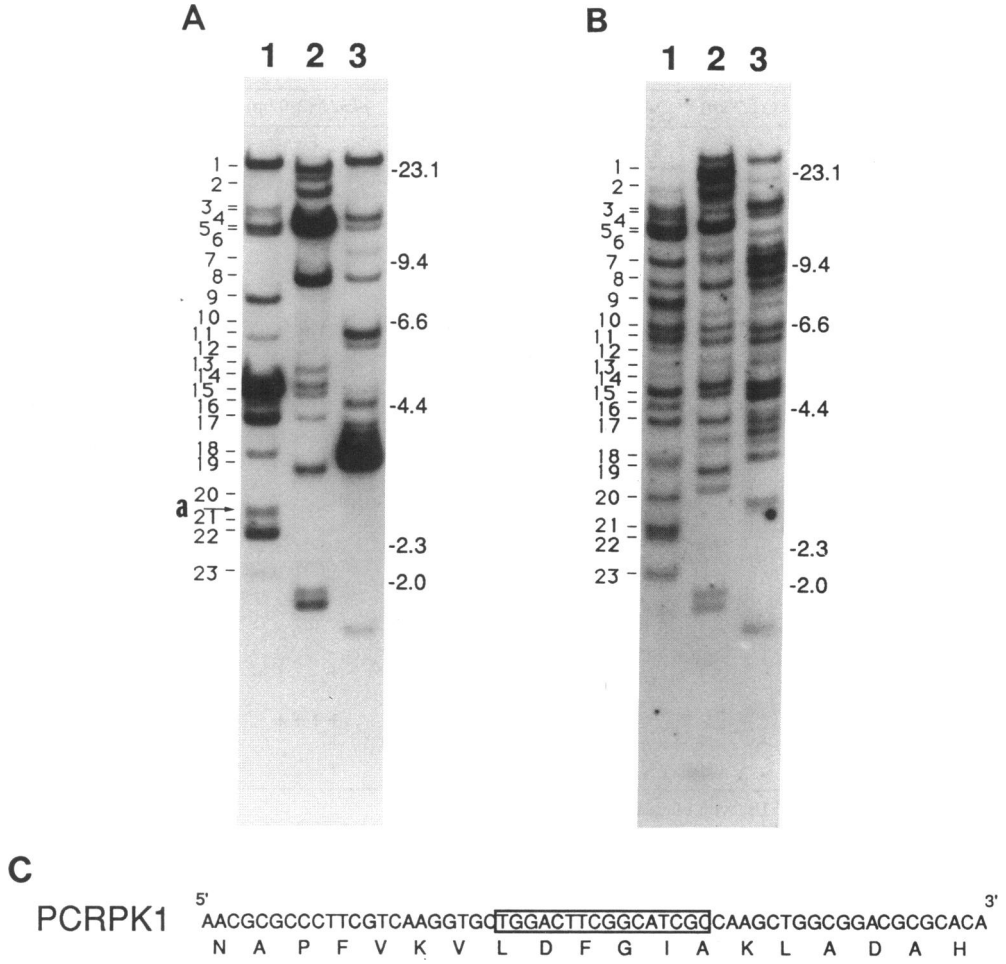

FIG. 1. Southern blot hybridization of the *M. xanthus* chromosomal DNA. (A) The 170-bp *Bam*HI fragment (PCRPK1) encompassing subdomains VIb, VII, and VIII of Pkn1 (9) was used as a probe. DNA fragments were nick-translated (10) with [α-^{32}P]dCTP, [α-^{32}P]dGTP, and [α-^{32}P]dATP and used as probes. Southern blot hybridization (13) with the nick-translated probes was carried out in 40% formamide at 42°C. The 4.9-, 12.4-, and 3.3-kb fragments are the *pkn1*-containing fragments in lanes 1, 2, and 3, respectively. (B) The 16-bp sequence shown in panel C (oligo3019) was used as a probe. Southern blot hybridization was performed at 42°C in a mixture consisting of 5× Denhardt's solution, 6× NET, 10% dextran sulfate, 0.5% sodium dodecyl sulfate, and 10^6 cpm of the probe per ml. The filter was washed with 2× SSC (1× SSC is 15 mM sodium citrate [pH 7.0] and 150 mM sodium chloride) at 50°C until λ DNA fragments, used as molecular standards, became negative. The probe was labeled at the 5′ end with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Boehringer Mannheim). For both panels, digestion with *Xho*I (lanes 1), *Pst*I (lanes 2), and *Sal*I (lanes 3) was used; the bands in lanes 1 are numbered on the left, and the numbers on the right represent the molecular sizes in kilobases. (C) DNA sequence of part of PCRPK1 and its deduced amino acid sequence. The identical 16-bp sequence found in PCRPK1, PCRPK2, and PCRPK3 is boxed.

were numbered, and their sizes are shown in Table 1. Since the intensities of the 23 bands were similar, except for bands 1, 2, 5, 6, 13, and 14, each DNA fragment probably contains a single putative *pkn* gene which contains a DNA sequence homologous with oligo3019 (Fig. 1B). The intensities of bands 5 and 6 were two times darker than those of bands of 15, 21, and 22 (*pkn1*, *pkn2*, and *pkn3*, respectively; see Table 1) which contained exactly the same sequence as oligo3019 used as probe. Therefore, the fragments corresponding to bands 5 and 6 probably contain more than one copy of the sequence hybridizing to oligo3019. On the other hand, the weak hybridization of bands 1, 2, 13, and 14 (Fig. 1B) are probably due to base mismatches in the homologous sequences.

The PCRPK1 probe used in Fig. 1A hybridized to only 12 of the 23 bands identified in Fig. 1B. However, an extra band (band a in Fig. 1A) was detected by PCRPK1 but not by oligo3019. This band (a 2.7-kb *Xho*I fragment) was cloned, and its partial sequence was determined. The deduced amino acid sequence of an open reading frame contained a sequence similarity with that of a conserved catalytic domain of eukaryotic protein serine-threonine kinases, consisting of 11 subdomains (data not shown). The open reading frame was thus designated *pkn11* (Fig. 2). However, there were two base mismatches between oligo3019 (Fig. 1C) and the *pkn11* sequence (the first T to C, and C at position 8 to T in oligo3019); this mismatch explains why band a was not detected in Fig. 1B.

PCRPK1 was used as a probe to screen for *pkn* genes in an *M. xanthus* genomic library present in λ phage. This λ phage library was constructed with use of the *M. xanthus* chromosomal DNA by the method previously described by Kohara et al. (7). Of 1,825 phages tested, 65 were positive, of which 40 were used for further characterization. The phage DNAs

TABLE 1. Identification of *M. xanthus* putative protein kinase genes

Band[a]	Size (kb)	Identification of indicated gene with probe (size)[b]:							Gene
		PCRPK1 (170 bp)	*pkn3* (1.7 kb)	*pkn5* (1.0 kb)	*pkn6* (0.6 kb)	*pkn11* (230 bp)	*pkn13* (1.8 kb)	*pkn15* (0.7 kb)	
1	~24.0	+++		+					
2	19.0				+				
3	15.5	+			+	+		+	
4	14.0		+	+	++	++			
5	12.7	++					++++	+	*pkn13*
6	12.0	++	+	+		+		+	
7	10.3		+		+	+			
8	8.7				+				
9	7.7	++			+	+	+		
10	6.7				+				
11	6.4	+	+		++++	++		+	*pkn6*
12	6.0								
13	5.7				+	+		+	
14	5.4				+	+		+	
15	4.9	++++	+		+				*pkn1*
16	4.5	+					+	++++	*pkn15*
17	4.2	+++	+		++	+	+	+	
18	3.5	+		++++	+		+		*pkn5*
19	3.4					+			
20	3.0								
21	2.55								*pkn2*[c]
22	2.5	+	++++						*pkn3*[c]
23	2.15	+			+	+			
a[d]	2.7	+				++++			*pkn11*
b	7.2					+			
c	4.0					+			

[a] Assignments of bands 1 to 23 are from Fig. 1B, with oligo3019 as probe.
[b] The DNA fragments used as probes were different sizes as indicated and were isolated from the individual *pkn* clones indicated. The band densities were visually quantitated and expressed as faint (+) to very strong (++++) bands. The band containing the gene used as the probe is indicated by ++++.
[c] The *pkn2* and *pkn3* genes were identified by PCRPK2 and PCRPK3, respectively, as probes.
[d] Band a hybridized with the PCRPK1 probe but not with oligo3019. Band a (see Fig. 1A) was identified as *pkn11*, and when *pkn11* was used as a probe, two new bands, b and c, were detected.

were digested with *Xho*I, and Southern blot analysis was performed with use of ^{32}P-labeled PCRPK1 and oligo3019 as probes. The 40 phages were classified into 18 groups on the basis of the patterns of restriction digests and the sizes of positive bands. The DNA fragments which hybridized with probes were isolated and cloned into the *Xho*I site of pUX(2) and pBluescript SK+ (Stratagene). Seven clones (*pkn2*, *pkn3*, *pkn5*, *pkn6*, *pkn11*, *pkn13*, and *pkn15*) were chosen for further characterization. From each clone, the DNA fragment that hybridized with oligo3019 or PCRPK1 was isolated, and its DNA sequence was determined. As shown in Fig. 2, all contained sequences homologous to Pkn1 in the region corresponding to subdomains VIb, VII, and VIII, indicating that all of them constitute a protein family. The subdomains VIb, VII, and VIII in eukaryotic protein kinases contain seven highly conserved amino acid residues, as shown at the top of Fig. 2 (3). Note that of these seven residues, six are conserved in the bacterial kinase family

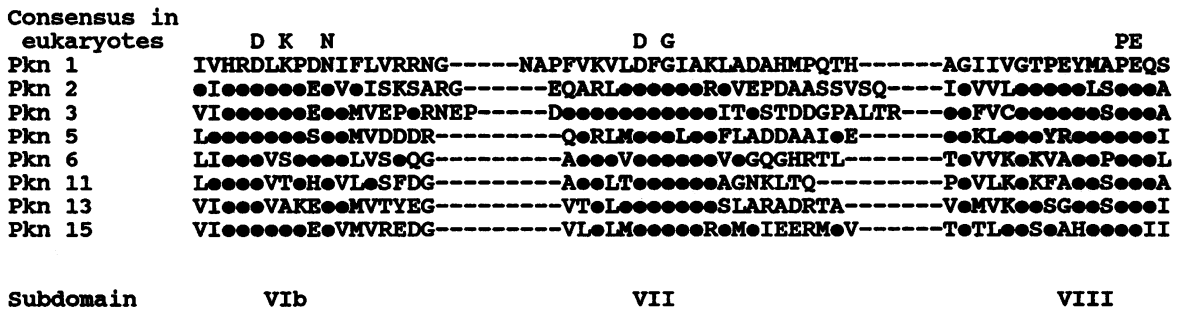

FIG. 2. Sequence comparisons among *M. xanthus* protein kinases. Phage DNA fragments containing subdomains VIb, VII, and VIII were identified by using nick-translated PCRPK1 and ^{32}P-labeled oligo3019 as probes and cloned into pUX(2) and pBluescript SK+ (Stratagene). To determine DNA sequences encompassing subdomains VIb, VII, and VIII, the fragments were further digested with various restriction enzymes and the resulting fragments were cloned into pUC9 (15). DNA sequencing was carried out by the chain termination method (11) with double-stranded plasmids. Only the regions for subdomains VIb, VII, and VIII are shown for Pkn1 (9), Pkn2, Pkn3, Pkn5, Pkn6, Pkn11, Pkn13, and Pkn15. Residues identical to Pkn1 are represented by solid circles. The subdomain sequences are separated by dashed lines. Highly conserved residues in eukaryotic protein serine-threonine kinases (3) are shown at the top of the figure.

(Fig. 2). From a crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase, it has been shown that subdomains VIb and VII form the catalytic loop and subdomain VIII plays a role in binding to an inhibitory peptide (6).

With DNA fragments from the cloned *pkn* genes as probes, the homologies between bands in Fig. 1B were further characterized by Southern blot hybridization (Table 1). The following fragments were used as probes: for *pkn3*, a 1.7-kb *Pst*I fragment consisting of subdomains I to XI; for *pkn5*, a 1.0-kb *Pst*I-*Xho*I fragment consisting of subdomains V to XI; for *pkn6*, a 0.6-kb *Sal*I-*Xho*I fragment consisting of subdomains IV to XI; for *pkn11*, a 230-bp *Rsa*I fragment consisting of subdomains IV to VIII; for *pkn13*, a 1.8-kb *Pst*I-*Eco*RI fragment consisting of subdomains III to XI; and for *pkn15*, a 0.7-kb *Pst*I-*Xho*I fragment consisting of subdomains I to XI. The results are summarized in Table 1. The *pkn2* and *pkn3* genes are those hybridized with PCRPK2 and PCRPK3 probes, respectively. Note that the *pkn11* probe hybridized to three new bands (bands a, b, and c; Table 1), which were not detected in the gels shown in Fig. 1B (band a contained the *pkn11* gene). In contrast to *pkn11*, there are some bands which hybridized with many different probes, such as bands 4, 6, 11, and 15. These *pkn* genes are probably more related to each other and form a putative kinase subfamily.

The present finding suggests that *M. xanthus* contains a large family of protein kinases consisting of at least 26 different genes. Recently, the DNA sequence of *pkn2* was determined, and its deduced amino acid sequence indicates that *pkn2* encodes a protein containing the catalytic domain with sequence similarities to eukaryotic protein serine-threonine kinases (8). Indeed, Pkn2 was found to be a protein threonine kinase required for normal differentiation of *M. xanthus* (8). It remains to be determined whether some of the genes identified above encode protein tyrosine kinases. The present results suggest that complex kinase cascades similar to those in eukaryotes might be involved in regulation of morphogenesis, cellular differentiation, and social behavior of this bacterium.

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