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An upstream activating sequence containing curved DNA involved in activation of the *Clostridium perfringens plc* promoter

Chieko Matsushita,[1] Osamu Matsushita,[1] Seiichi Katayama,[1] Junzaburo Minami,[1] Kenichi Takai[2] and Akinobu Okabe[1]

Author for correspondence: A. Okabe. Tel: +81 878 98 5111. Fax: +81 878 98 7109. e-mail: microbio@kms.ac.jp

[1] Department of Microbiology, Kagawa Medical School, 1750-1, Miki-cho, Kita-gun, Kagawa 761-07, Japan

[2] Okayama Central Hospital, 2-18-19, Hokan-cho, Okayama 700, Japan

The *plc* gene, which encodes phospholipase C (α-toxin) of *Clostridium perfringens*, possesses three poly(A) tracts forming an intrinsically curved DNA region immediately upstream of the promoter. The *in vivo* transcriptional activity of the plasmid-borne *plc* gene was stimulated by this curved-DNA-containing sequence, depending on its proper linear and rotational orientation. The *in vitro* transcriptional activity of the *plc* gene was also stimulated by the upstream sequence. In addition, the stimulatory effect of the sequence and the degree of DNA bending were greater at lower temperature, as was demonstrated by both *in vitro* and *in vivo* transcription assays, and a gel-mobility assay, respectively. A similar temperature effect was also observed with the chromosomal *plc* gene. These observations suggest that the upstream DNA curvature *per se* stimulates the initiation of transcription of the *plc* gene, possibly through direct contact with RNA polymerase.

Keywords: curved DNA, gene expression, *Clostridium perfringens*, phospholipase C

INTRODUCTION

Clostridium perfringens is a spore-forming anaerobic bacterium which is widely distributed in nature, and also lives in the large intestines of humans and other animals. It sometimes causes histolytic infections such as gas gangrene upon infecting a host. Since phospholipase C (PLC; EC 3.1.4.3) is the most important virulence factor of the organism (Hatheway, 1990; Titball, 1993; Ninomiya *et al.*, 1994), PLC production seems to be critically important to the host–parasite interrelationship. Although the PLC-encoding gene (*plc*) has been cloned and sequenced (Leslie *et al.*, 1989; Okabe *et al.*, 1989; Saint-Joanis *et al.*, 1989; Titball *et al.*, 1989; Tso & Siebel, 1989), the regulation of *plc* gene expression is not well understood.

In a previous paper (Toyonaga *et al.*, 1992), we reported that three poly(A) tracts, with a 10 or 11 bp periodicity located immediately upstream of the *plc* promoter (P_{plc}) form an intrinsic DNA curvature. Since intrinsically curved DNA located upstream of a promoter enhances the activities of downstream promoters (Pérez-Martín *et al.*, 1994), it seems likely that expression of the *plc* gene is also stimulated by the curved DNA. To prove this, we examined the *in vivo* effect of the upstream curved DNA on the transcriptional activity of P_{plc} using two sets of mutant plasmids, one with deletions of the poly(A) tracts, and the other with oligonucleotide inserts of various lengths between the tracts and the −35 sequence.

Curved DNA containing-fragments show anomalously slow electrophoretic mobility at low temperature. This gel migration anomaly has been suggested to be due to conformational changes of the curved DNA induced by a decrease in temperature (Diekmann, 1987; Koo & Crothers, 1988). Therefore, it is conceivable that the curved DNA *per se* modulates the promoter activity in response to changes in temperature. In order to assess this possibility, *C. perfringens* strain 13 PLC^- containing plasmid-borne *plc* genes with and without the curved DNA was grown at various temperatures, and then the levels of *plc* transcripts in the cultures were determined. We also examined the *in vitro* transcriptional activity of these *plc* genes at different temperatures to rule out the possible involvement of a DNA-binding protein in the observed temperature-dependent stimulatory effect.

This paper describes the stimulatory effect of the curved-DNA-containing upstream sequence on P_{plc} activity and

Abbreviations: PLC, phospholipase C; P_{plc}, promoter of the *plc* gene.

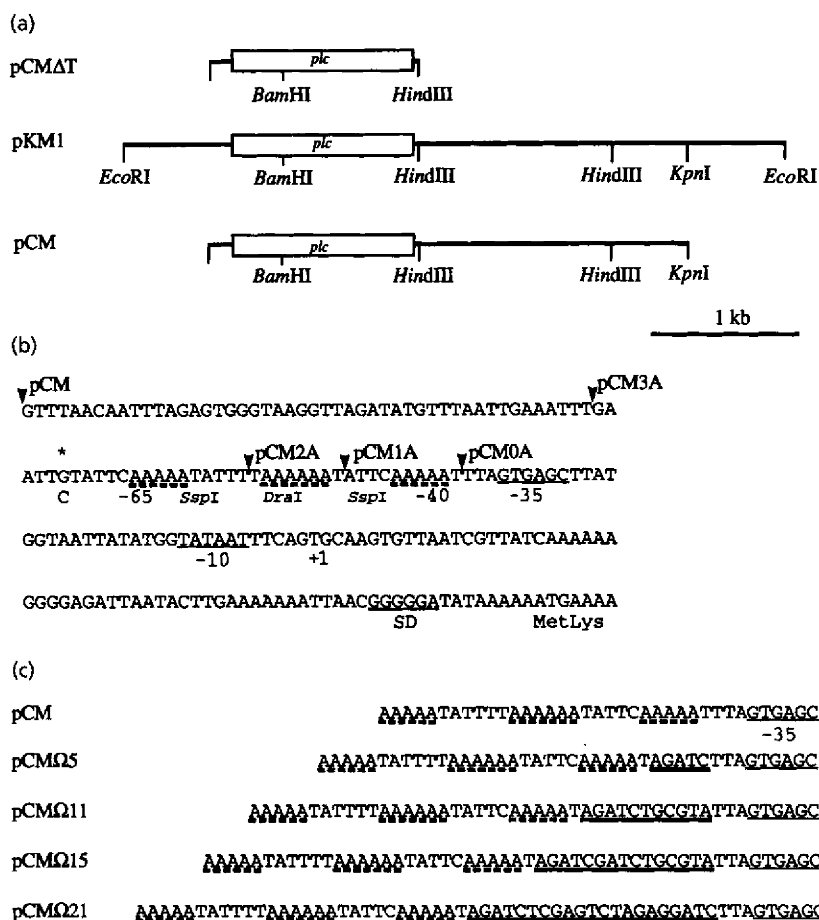

Fig. 1. (a) Partial physical maps of *plc*-containing fragments cloned into pCMΔT and pCM. pCMΔT was constructed by cloning a 1·5 kb fragment, of which the 5′ end was located at the position indicated by pCM in (b), into pJIR418. pCM was constructed by replacing the region from the *Bam*HI to *Hin*dIII sites of pCMΔT with the region from the *Bam*HI to *Kpn*I sites of pKM1. (b) Nucleotide sequence of the *plc* promoter and its upstream regions. The nucleotide numbers are assigned with reference to the transcription start site [+1 (Titball *et al.*, 1992)]. The deletion endpoint of each deletion plasmid is indicated by a downward arrowhead. An asterisk indicates the nucleotide substituted by site-directed mutagenesis to generate an *Eco*RI site. The three poly(A) tracts are underlined with dashes. (c) Nucleotide sequences of plasmid derivatives with insertions of 5 to 21 bp nucleotides. The number of nucleotides inserted into pCMΩ5, pCMΩ11, pCMΩ15 and pCMΩ21 were 5, 11, 15 and 21, respectively. The inserts are underlined with thick lines.

modulation of the effect through its conformational change in response to a change in temperature.

METHODS

Bacterial strains, plasmids and growth conditions. The *C. perfringens* strains used in this study were NCTC 8237 (Okabe *et al.*, 1989), and strain 13 PLC⁻ (Kameyama *et al.*, 1996). The *plc* gene of the latter strain is disrupted by homologous recombination with pKMB141, a derivative of pJIR418, which lacks *oriCP* and contains a 376 bp *Fok*I fragment of the *plc* gene. *Escherichia coli* strain DH5α (Hanahan, 1985) was used to construct plasmid derivatives. *E. coli* BMH 71-18 *mutS*, which was used for site-directed mutagenesis, was obtained from Clontech Laboratories. Plasmid pJIR418 (Sloan *et al.*, 1992) was used as an *E. coli*–*C. perfringens* shuttle plasmid. The following two plasmids were used to construct pJIR418 derivatives: pCMΔT, pJIR418 into which a 1·5 kb fragment containing the *plc* gene was cloned from *C. perfringens* NCTC 8237 (Toyonaga *et al.*, 1992); and pKM1 (Okabe *et al.*, 1989), pBR322 into which a 4·4 kb *Eco*RI fragment containing the *plc* gene was cloned from NCTC 8237. *C. perfringens* strains were grown anaerobically in GAM broth (Nissui Pharmaceutical) with and without 10 μg chloramphenicol ml^{-1} and 20 μg erythromycin ml^{-1} (Matsushita *et al.*, 1994a). *E. coli* strains were grown in Luria–Bertani broth (LB broth; Gibco) as described previously (Matsushita *et al.*, 1994b).

Site-directed mutagenesis. This was performed using a Transformer site-directed mutagenesis kit (Clontech), according to the instruction manual of the manufacturer. An oligonucleotide containing a *Stu*I site, 5′ GACTTGGTTGAGGCCTCACCAGTCAC 3′, was used as a selection primer. The following mutagenic primers were used to generate new restriction sites: (1) 5′ GAAATTTGAATTCTATTCAAAAATA 3′ for generation of a new *Eco*RI site between nucleotide positions −77 and −72 relative to the *plc* transcriptional start; and (2) 5′ AAAAAATATTCAAAAATAGATCTTAGTGAGCTTATG 3′ and 5′ AAAAAATATTCAAAAATAGATCTGCGTATTAGTGAGCTTATG 3′ for insertion of 5 and 11 nucleotides, respectively, which contained a *Bgl*II site between nucleotide positions −39 and −38 relative to the *plc* transcriptional start. A DNA fragment prepared by annealing two synthetic oligonucleotides, 5′ GATCTCGAGTCTAGAG 3′ and 5′ GATCCTCTAGACTCGA 3′, was also used to insert 16 nucleotides into the newly generated *Bgl*II site.

Construction of mutant plasmids. pCMΔT derivatives with deletions and insertions in the *plc* upstream region were constructed by cutting and rejoining at restriction sites of pCMΔT and those generated by site-directed mutagenesis, and also by inserting synthetic oligonucleotides (see Fig. 1). Since pCMΔT and its derivatives did not contain a *plc* transcriptional terminator, the 3′ end of the insert DNA in each construct was extended further downstream by replacing a 1·0 kb *Bam*HI–*Kpn*I fragment of each construct with a 2·8 kb *Bam*HI–*Kpn*I fragment of pKM1 containing the *plc* terminator (Fig. 1a). The set of pCM derivatives containing different numbers of poly(A) tracts was designated as follows (see Fig. 1b): pCM, pCM3A, pCM2A, pCM1A and pCM0A. The other set of pCM derivatives

constructed with the insertion of oligonucleotides (see Fig. 1c) are called pCMΩ5, pCMΩ11, pCMΩ15 and pCMΩ21.

Determination of *plc* mRNA levels. When cultures of *C. perfringens* strains reached an OD_{600} of 0·9–1·0, total RNA was prepared as described elsewhere (Katayama *et al.*, 1993). Northern hybridization was performed as described previously (Matsushita *et al.*, 1994b). The levels of *plc* transcripts were determined by quantitative Northern analysis using various amounts (0·125–1 μg) of RNA as described previously (Tsutsui *et al.*, 1995).

Nucleotide sequencing and DNA manipulations. *C. perfringens* strains were transformed by a modification of the method of Allen & Blaschek (1990). Restriction endonucleases and DNA modifying enzymes were purchased from Takara Shuzo, Toyobo and New England Biolabs. All recombinant DNA procedures were performed as described by Sambrook *et al.* (1989). The nucleotide sequence of each plasmid construct was determined as described previously (Matsushita *et al.*, 1994b).

Gel mobility assay. To analyse the mobility of a curved-DNA-containing fragment, 5% (w/v) polyacrylamide [acrylamide/bisacrylamide = 29·2:0·8 (w/w)] gel electrophoreses were performed in 45 mM Tris/borate, pH 8·0 and 1 mM EDTA at 6 V cm^{-1}. The temperature during the electrophoresis was kept constant (25±0·5 or 45±0·5 °C) by means of an apparatus with a device for temperature regulation [Koike Precision Instruments, Kawasaki; model E-IES 17–20TR (Matuo *et al.*, 1982)]. Two plasmids, pCMΔT and pCMΔT0A, were used as the source of DNA containing the curved DNA and a control, respectively. Digests of these plasmid DNAs with *Pvu*II and *Nhe*I were electrophoresed. *Hin*fI-digested pBR322 DNA was used as the unbent molecular mass marker. Semi-log plots of the relative gel mobilities of the fragments were calibrated by least-squares fitting. The apparent length of each fragment was then calculated from the least-squares calibration plot. The gel migration anomaly is presented in terms of R_L, which is defined as the ratio of the apparent to true fragment length.

Assays for PLC. Phospholipase C activity in the culture supernatant was assayed by the method of Kurioka & Matsuda (1976) using *p*-nitrophenylphosphorylcholine (Sigma) as a substrate. Cellular protein concentration was determined as described previously (Katayama *et al.*, 1993).

***In vitro* transcription assay.** Template DNAs were prepared by digesting pCMΔT and pCMΔT0A with *Pvu*II and *Acc*II, followed by electroelution of 335 and 245 bp fragments, respectively. RNA polymerase was prepared from *C. perfringens* NCTC 8237 cells as described by Garnier & Cole (1988). The *in vitro* transcription assay was performed as described elsewhere (Garnier & Cole, 1988) except that 40 ng DNA template and 1 μg RNA polymerase were used. The relative amounts of the *plc* transcripts were determined with a Fuji BAS1000 Bio-image analyser (Fuji Photo Film).

RESULTS

Effects of deletions and insertions on the *plc* promoter activity

In order to determine whether or not the poly(A) tracts immediately upstream of P_{plc} stimulate the transcriptional activity of P_{plc}, pCM derivatives with deletions and insertions in the *plc* upstream region were constructed (Fig. 1). The identities of all mutations generated were confirmed by nucleotide sequencing of each construct. The DNA curvature present in the derivatives with deletions was examined by determining the gel mobilities of fragments containing poly(A) tracts at the centre. The R_L value decreased as the number of residual poly(A) tracts decreased (data not shown). All the pCM derivatives were introduced into *C. perfringens* strain 13 PLC⁻ and then *in vivo* transcription of the plasmid-borne *plc* genes was examined. When the three poly(A) tracts were deleted successively from the 5′ end, the level of *plc* mRNA decreased markedly (Fig. 2a). Deletion of the first poly(A) tract decreased the P_{plc} activity to about 30% of the level of the wild-type having the three tracts. Deletion of the second and third tracts caused further decreases in the transcriptional activity, which finally fell to 10% of the wild-type level.

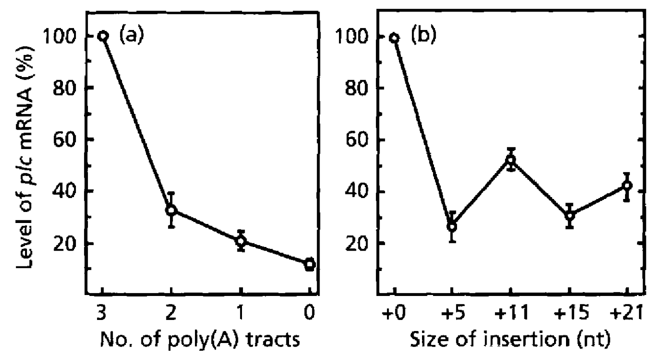

Fig. 2. Effects of (a) deletion of poly(A) tracts and (b) insertion of 5 to 21 bp between the curved DNA and the −35 sequence on transcription of the *plc* gene. *C. perfringens* strain 13 PLC⁻ harbouring pJIR418 and its derivatives were grown in GAM broth with antibiotics at 37 °C. RNA was extracted when the cultures reached an OD_{600} of 0·9–1. The relative amounts of *plc* mRNA were determined by quantitative Northern analysis, as described in Methods. Data are expressed as mean±SD (bars) for triplicate determinations.

The number of tracts determines not only the bend angle, but also the span and the centre of the bend. Therefore, the question was raised as to whether or not the position of the bend is another important factor for the stimulatory effect. To assess this possibility, we examined the effect of insertional mutations on the *plc* gene expression (Fig. 2b). All the inserts resulted in a marked decrease in the transcriptional activity of P_{plc}. Furthermore, the mutant promoters in which the curved DNA was rotated by one or two turns of the helix (i.e. 11 and 21 bp insertions) and was thus in the same phase as the wild-type, showed significantly higher activity than those in which the curved DNA was in the opposite phase (i.e. 5 and 15 bp insertions). Therefore, appropriate placement of the curved DNA in terms of both linear and rotational orientation is required for its stimulatory effect on P_{plc}.

P_{plc} activities at different temperatures

Curved-DNA-containing fragments exhibit anomalously slow mobility in an electric field at low temperature (below 6 °C), but not at high temperature (above 50 °C). A more subtle change in the molecular shape of the

Table 1. **Comparison of the transcriptional activity at various temperatures between the wild-type and mutant *plc* genes**

All assays were performed in triplicate using 0·125–1 μg RNA as described in Methods.

Temp. (°C)	Wild-type/mutant transcription ratio	
	in vivo*	in vitro†
25	8·6 ± 0·4	> 9·3 ± 0·1‡
30	7·6 ± 0·5	7·3 ± 0·9
37	6·6 ± 0·4	6·3 ± 0·6
41	4·5 ± 0·4	ND
45	3·8 ± 0·2	3·9 ± 0·8

ND, Not determined.

* The PLC⁻ strain harbouring pCM (wild-type) or pCM0A (mutant) was grown at the indicated temperatures. The relative amounts of *plc* mRNA were determined in each culture. Values are expressed as the ratio of the relative amount of *plc* mRNA in pCM-containing cells to that in pCM0A-containing cells.

† Values are expressed as the ratio of the relative amount of *plc* mRNA transcribed from the pCM-derived template to that from the pCM0A-derived one.

‡ The amount of *plc* mRNA was lower than the lowest value determined by the method.

curved DNA may be induced by changes in temperature in a narrower range, as has been demonstrated by Diekmann (1987) for synthetic curved DNAs. We performed gel mobility analyses of the curved DNA at 25 and 45 °C, two growth-permissive temperatures. The R_L values of a 337 bp fragment with the curved DNA determined at 25 and 45 °C were 1·29 ± 0·01 and 1·09 ± 0·01, respectively, while those of a 247 bp fragment without the curved DNA at the two temperatures were both nearly 1 (1·08 ± 0·00 at 25 °C and 1·03 ± 0·00 at 45 °C). This clearly indicates the conformation of the curved DNA changes even in such a narrow temperature range.

In order to determine the stimulatory effect of the curved DNA at different temperatures, we grew *C. perfringens* strain 13 PLC⁻ carrying pCM, a wild-type plasmid, at various temperatures between 25 and 45 °C, and then determined the level of *plc* mRNA in each culture. The level of *plc* mRNA decreased as the growth temperature increased. In contrast, in the case of pCM0A, a mutant plasmid without the curved DNA, it was nearly constant at all the temperatures examined (data not shown). Since the RNA contents of cultures growing rapidly at high temperature may differ from those growing slowly at low temperature, the *plc* mRNA levels in the two different cultures grown at the same temperature were compared with each other (Table 1). The ratio of the mRNA level in the pCM-carrying cells to that in pCM0A-carrying ones increased with decreasing temperature. These results suggest that *plc* is expressed efficiently at lower temperatures, probably through the formation of a more suitable conformation of the curved DNA.

In vitro transcription of the *plc* gene

There is a possibility that a protein binding to the upstream region is involved in the effect of the curved DNA observed in the *in vivo* experiment. To eliminate this, the *in vitro* transcriptional activity of the wild-type *plc* gene from pCM was compared with that of the mutant gene from pCM0A. The relative amounts of *plc* transcripts from the former were higher than those from the latter at all the temperatures examined (Table 1). Moreover, the stimulatory effect of the curved DNA on the transcriptional activity was more prominent at low temperature than at high temperature (Table 1).

Expression of the chromosomal *plc* gene at different temperatures

To ascertain whether or not the chromosomal *plc* gene is also expressed in a temperature-dependent fashion, *C. perfringens* NCTC 8237 was grown at various temperatures, and then the levels of *plc* mRNA and PLC activity were determined. As shown in Table 2, lowering of the growth temperature increased the level of *plc* mRNA. PLC production was also higher at low temperature than at high temperature. The temperature effect on PLC production was not so prominent as that on *plc* gene transcription, which may be due to depression of the synthesis and secretion of PLC at low temperature.

DISCUSSION

The results presented here clearly indicate that a promoter upstream sequence with curved DNA stimulates the promoter activity of the *plc* gene which encodes PLC, a principal virulence factor of *C. perfringens*. They also indicate that the curved DNA in the sequence modulates it in a temperature-dependent manner. The promoter upstream DNA curvature has been postulated to facilitate the formation of the closed complex and thereby stimulate promoter activity (Pérez-Martín *et al.*, 1994). However, this remains dubious because the A + T-rich binding site for the RNA polymerase α subunit (spanning positions −40 to −60), which functions as a third promoter recognition element (Ross *et al.*, 1993; Rao *et al.*, 1994), overlaps the DNA curvature. The third promoter recognition element may contribute partly to the stimulatory effect. However, the DNA curvature can be assumed to play an essential role in the stimulatory effect on *plc* gene expression for the following reasons. First, the stimulatory effect depends largely on the first poly(A) tract between positions −62 and −66, as demonstrated by the deletion experiment. Second, the transcriptional activity of the *plc* gene is modulated by a change in temperature, correlating with the conformational change of the curved DNA, as was observed by gel-mobility analysis. Our interpretation is supported by the recent finding that curved DNA interacts with RNA polymerase further

Table 2. Levels of *plc* mRNA and PLC activity in *C. perfringens* cultures grown at various temperatures

C. perfringens NCTC 8237 was grown to an OD_{600} of 0·9–1·0 at the indicated temperatures. The levels of *plc* mRNA and PLC activity were determined in two separate experiments. The values are the mean ± SD for triplicate determinations.

Temp. (°C)	*plc* mRNA* (%)	PLC activity†	
		[(ml culture)$^{-1}$]	[(mg cellular protein)$^{-1}$]
25	100	1·10 ± 0·00	6·51 ± 0·01
30	85 ± 2·5	ND	ND
37	47 ± 2·5	0·87 ± 0·02	4·92 ± 0·11
41	31 ± 1·5	ND	ND
45	23 ± 0·5	0·53 ± 0·02	2·63 ± 0·06

* Relative amounts of *plc* mRNA are expressed as percentages of that in cells grown at 25 °C.

† PLC activity in the culture supernatant was determined using *p*-nitrophenylphosphorylcholine as a substrate and expressed as nmol substrate hydrolysed min^{-1}.

ND, Not determined.

upstream of the third element, forming a stable complex (Nickerson & Achberger, 1995).

In the previous study (Toyonaga *et al.*, 1992), we cloned the *plc* gene into a multi-copy plasmid, pUC19, and expressed it in *E. coli*, a heterologous host. We found that the synthesis of PLC by *E. coli* increased when the upstream region containing the poly(A) tracts was deleted (Toyonaga *et al.*, 1992). The discrepancy seems to be due to the difference in the host/vector system in this and previous studies. This intrinsic DNA curvature present in the *bla* gene of pUC19 might indirectly affect expression of the cloned *plc* gene, as in the case of the curved DNA present in the *luxAB* reporter system (Forsberg *et al.*, 1994).

Many pathogenic bacteria regulate the expression of their virulence factors in response to changes in various environmental parameters such as temperature, osmolarity, ions, oxygen and pH, all of which could be used as signals to detect entry into host tissues (Mekalanos, 1992). The transition from ambient low temperature to body temperature induces virulence genes in several organisms, e.g. genes of *Shigella flexneri* involved in eukaryotic cell invasion and those of *Pseudomonas aeruginosa* in alginate capsule synthesis (Mekalanos, 1992). On the contrary, the intrinsic DNA curvature of the *plc* gene seemingly exerts an opposite effect in terms of the thermoregulation of virulence genes. *C. perfringens* is potentially highly virulent but usually avirulent, living as a commensal bacterium in the large intestines of humans and other animals. This organism can be expected to contribute to carcass putrefaction through the concerted actions of a variety of hydrolytic enzymes including PLC. Thus, to fulfil its commensal and saprophytic life styles, efficient expression of the *plc* gene at low temperature may be advantageous for the organism.

The present study focused on the function of the immediately adjacent upstream curved DNA in P_{plc} activity. Although curved DNA *per se* is unambiguously a *cis*-acting element activating P_{plc} and contributes to the thermoregulation of the *plc* gene, the *plc* gene seems to be at least partly controlled by a complex mechanism involving *trans*-acting factors (Katayama *et al.*, 1993) and a global-regulatory network (Rood & Lyristis, 1995) governed by a two-component (VirR/VirS) system (Lyristis *et al.*, 1994; Shimizu *et al.*, 1994). Such regulatory systems would play a key role in pathogenicity of the organism upon infecting the host. Work aimed at the identification of *trans*-acting factors for the *plc* gene is now in progress.

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