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On 17th November 2014 at Double Tree by Hilton Hotel Chicago - North Shore , USA

Function of the phased A-tracts upstream of the phospholipase C gene promoter in *Clostridium perfringens*

Seiichi Katayama

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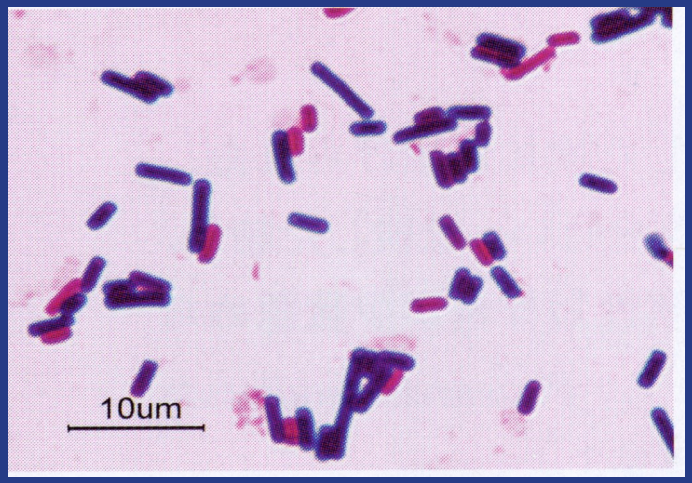
Introduction

Clostridium perfringens



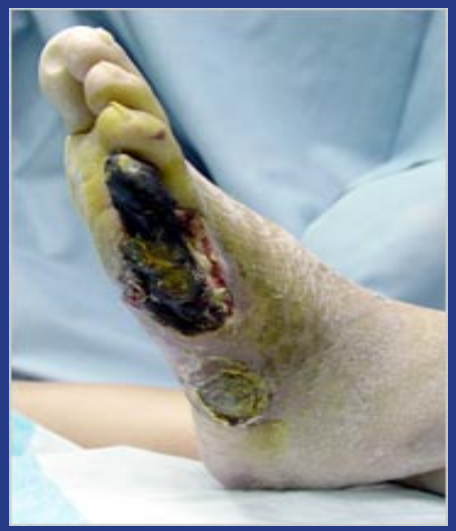
- Gram positive rod
- Spore-forming
- Obligate anaerobe
- Living in animal intestinal tracts and soil
- Pathogen for humans and animals

gas gangrene ← α-toxin

food poisoning ← enterotoxin

Gas gangrene

- Wound, injury of surgery → infection → α-toxin (phospholipase C) produced by *C. perfringens* → destructions of cell membranes → damages of tissues
- Treatment → Cut open of the wound
  Antibiotics
  High-pressure oxygen



α–toxin (*plc*) gene expression (NCTC8237)

Table 1. Levels of *plc* mRNA and PLC activity in *C. perfringens*

Temperature (° C)	*plc* mRNA (%)	PLC activity (nmol/min/mg/cellular protein)	Ratio (%)
25	100	6.51 ± 0.01	100
37	47 ± 2.5	4.92 ± 0.11	75.6
45	23 ± 0.5	2.63 ± 0.06	40.4

The *plc* gene expression increased at lower temperatures.

The phased A-tracts upstream of the *plc* gene promoter

The phased A-tracts upstream of phopholipase C (*plc*) gene promoter

NCTC8237 (=ATCC13124)

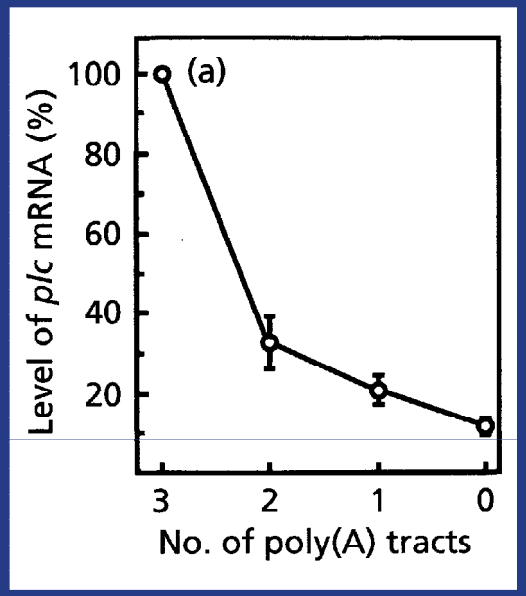
-79 **3 phased A-tracts (-66 to -44)** -35 ***plc* promoter** -10 +1

TTGAATTGTATTCAAAAATATTTTAAAAAATATTCAAAAATTTAGTGAGCTTATGGTAATTATATGGTATAATTTCAGTG

- The phased A-tracts are almost conserved among *C. perfringens* strains.
- What is the effect of the phased A-tracts on the *plc* gene expression?

plc gene expression *in vivo*

C. Perfringens PLC⁻ strain



3 phased A-tracts (–66 to –44) *plc* promoter –35

3Ap 5'-TATTCAAAAATATTTTAAAAAATATTCAAAAATTTAGTGAGC-3'

0Ap 5'-CGTTGTAAAACGACGGCCAGTGCCAAGCTGATCTTAGTGAGC-3'

- The A-tracts promoted the *plc* gene expression, *in vivo*.

[Matsushita, *et al*. *Microbiology* **142**: 2561-2566. 1996]

Promoter competition assay 1

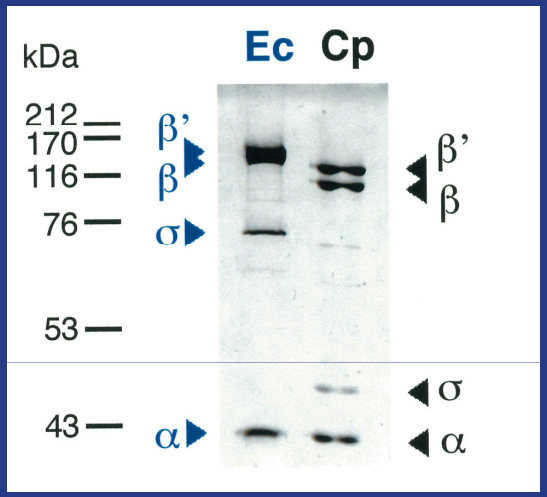


Fig. 1 Purified RNA polymerase.
Ec : *Escherichia coli*, Cp: *C. perfringens*

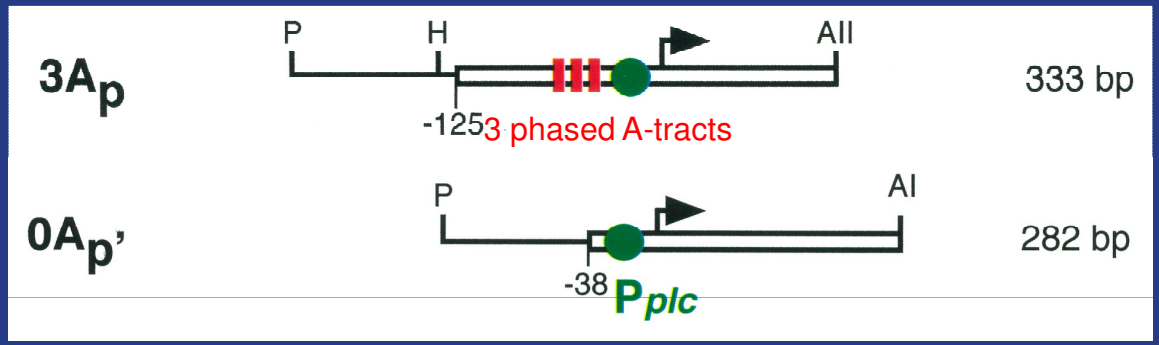
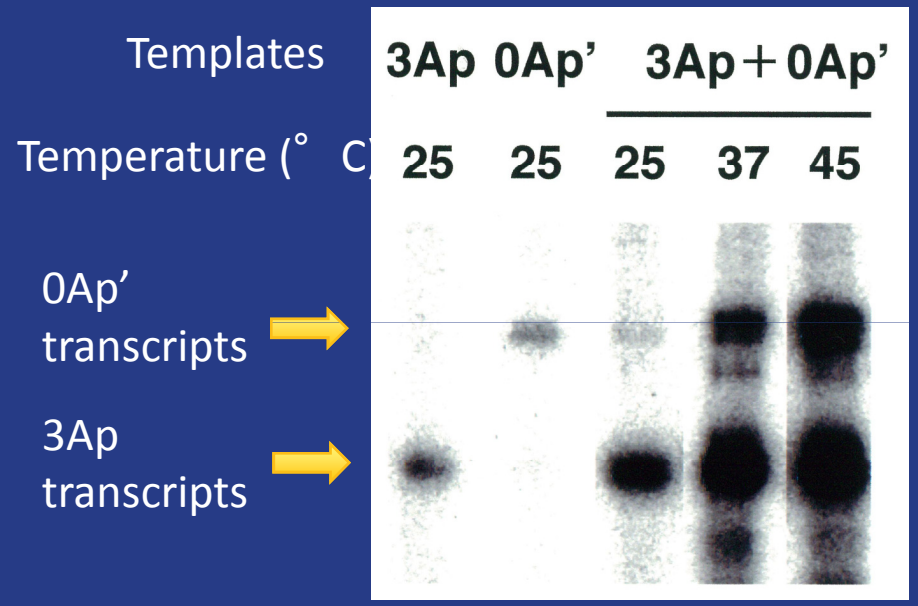


Fig. 2 Template DNAs used
in promoter competition assays.

- *In vitro* transcription with two promoters on DNA fragments, RNA polymerase, and NTPs was done.

Promoter competition assay 2

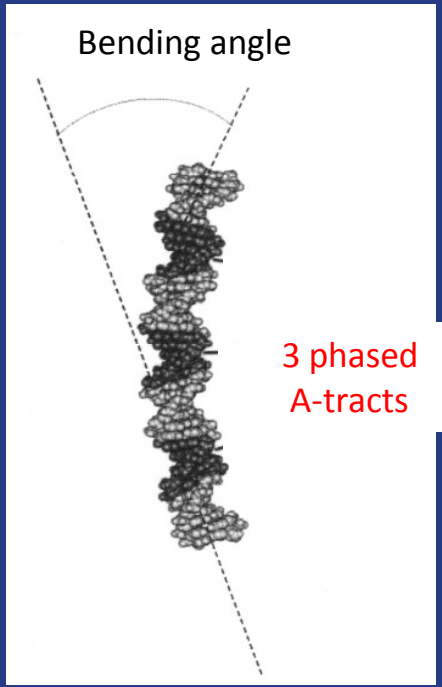


Temperature (° C)	Ratio of mRNA levels (3Ap/0Ap')
25	30.8 ± 4.3
37	4.6 ± 0.8
45	1.9 ± 0.2

- The phased A-tracts enhanced the *plc* gene expression at lower temperatures.

[Katayama, *et al*. *EMBO J* **18**:3442-3450, 1999]

Phased A-tracts can bend



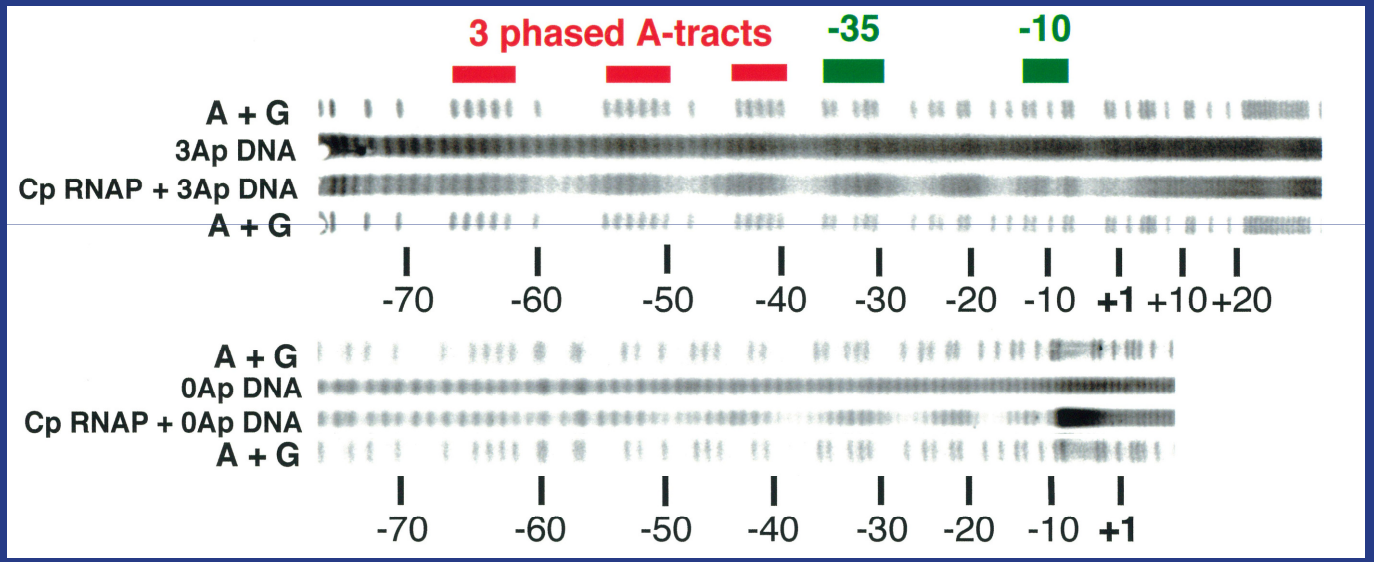
The bending angle of 3 phased A-tracts

Temperature (° C)	Bending centre (bp)	Bending angle (°)
15	-17 ± 2	46.6 ± 1.3
25	-25 ± 1.3	40.3 ± 0.8
37	-29 ± 5.2	35.9 ± 0.6

- The 3 phased A-tracts can bend at lower temperatures.

[Katayama, *et al*. Unpublished data]

Hydroxyl radical footprinting



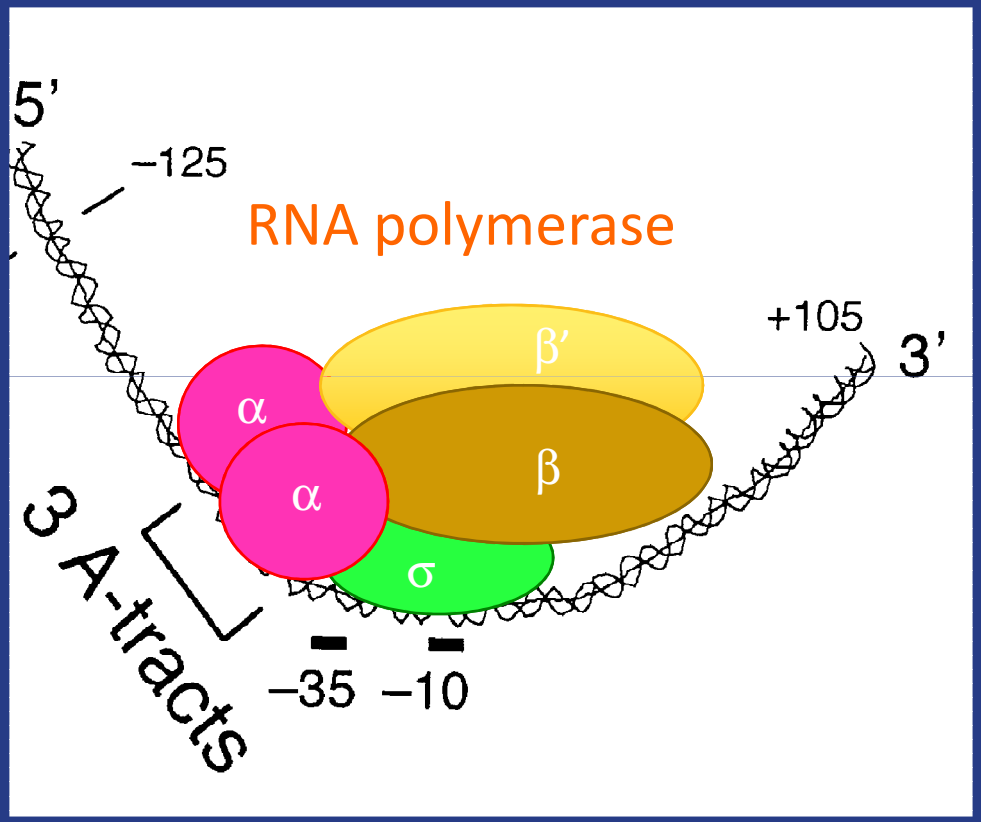
Protected region (bp)

-64 to -1

-54 to -1

- 3 phased A-tracts extended the contact region with RNA polymerase.

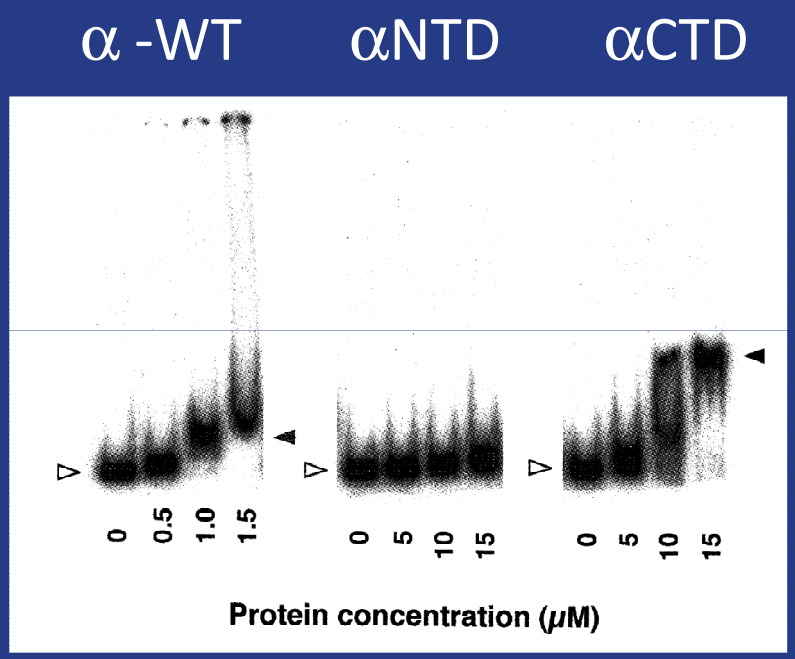
Scheme of contact of RNA polymerase with the phased A-tracts



➡ α subunits bind to the A-tracts?

Binding of the α subunits of *Cp* RNA polymerase to the phased A-tracts

Gel shift assay for binding of the *Cp* α subunits to 3A DNA

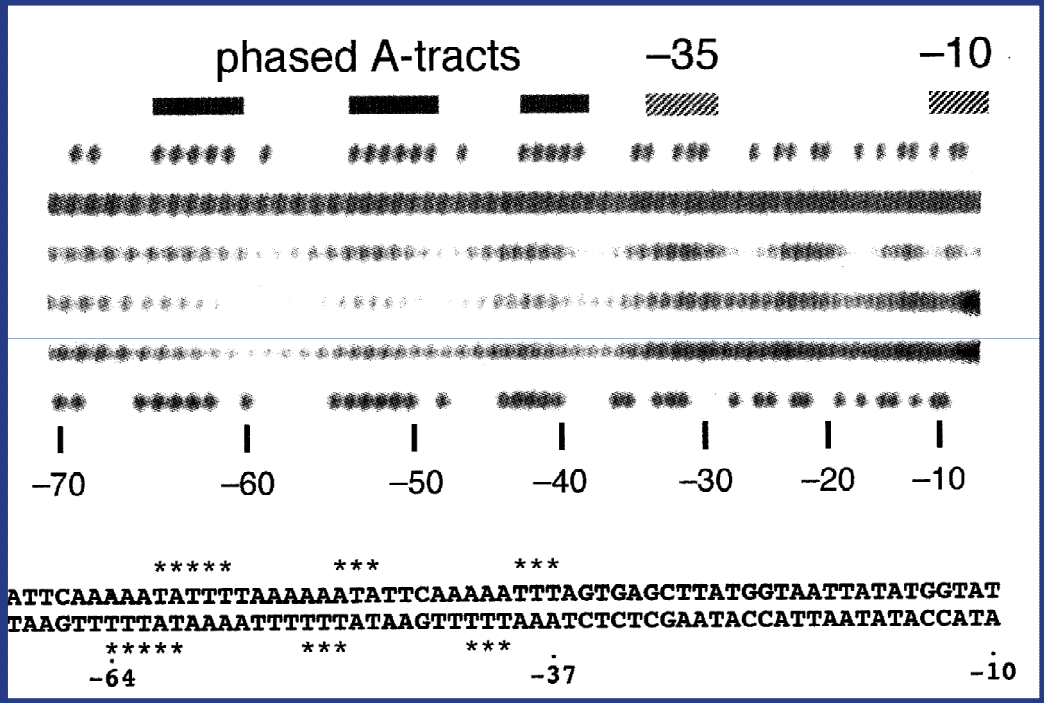


- The C-termnal domain of the α subunit (αCTD) of *Cp* RNA polymerase bound to the phased A-tracts.

[Katayama, *et al*. *FEBS Lett* **509**: 235-238, 2001]

Hydroxyl radical footprinting

A/G
DNA
DNA +*Cp* RNAP
DNA + α subunit
DNA + α CTD
A/G



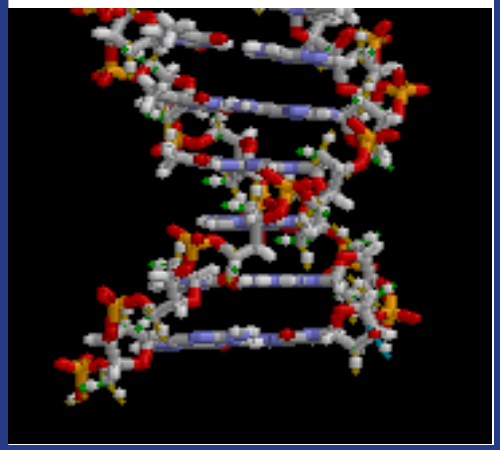
*Protected nucleotides by α subunits of *Cp* RNAP

- *Cp* α subunits and α CTD protected the region of the phased A-tracts.

Chemicals binding to DNA

Minor groove

Major groove

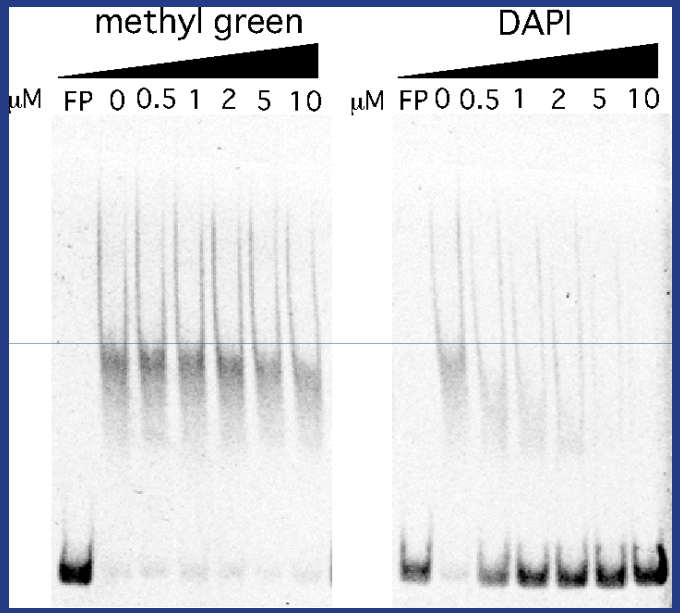


DAPI
(4’ 6-diammidino-2-phenyllindole)

Methyl green

Structure of DNA

Gel shift assay using methyl green and DAPI



FITC-3Ap DNA 25 nM,
Cp α-WT 4 μM
+ inhibitor
⇩
Incubation 25℃, 30 min
⇩
5% PAGE

FP:free probe

- DAPI inhibited the binding of the α subunit to the phased A-tracts.
- The α-CTD binds to the minor groove of 3A.

Affinity of the phased A-tracts to the α subunits of *Cp* RNA polymerase

Table 3. Affinity of *C. perfringens* α subumit to 3A or 0A DNA

DNA (25 μM)	Dissociation constant* *K*d （M)	Ratio
3A	$6.1 \pm 0.3 \times 10^{-8}$	1.0
0A	$1.5 \pm 0.1 \times 10^{-6}$	24.1

*Measuerd by surface plasmon resonance (SPR)

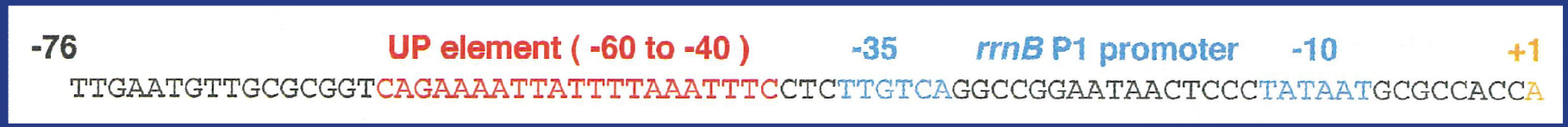
[Katayama, *et al*. *Anaerobe* **23**: 62-69, 2013]

The affinity was of the same order magnitude as that of H-NS proteins (*E. coli*) binding to a DNA fragment containing A_5A_6 sequence (*K*d = 2.7×10^{-8} M), measured by SPR.

[Bouffartgues, *et al. Nucleic Acids Research* **35**:e39, 2007.]

The contact path of the α subunit of *C. perfringens* RNA polymerase with the phased A-tracts

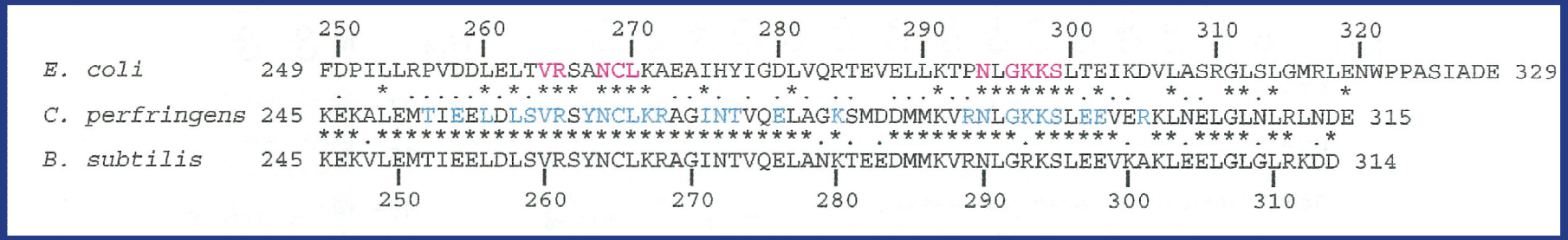
UP element of *E. coli*



- Upstream (UP) element is an A/T rich sequence upstream of the *rrnB* P1 promoter (16S rRNA gene).
- UP element enhances the promoter activity, which contacts with αCTD of *Ec* RNA polymerase.

[Ross, *et al*. *Science* **262**:1407-1413. 1993.]

The positions of alanine substitutions in αCTD

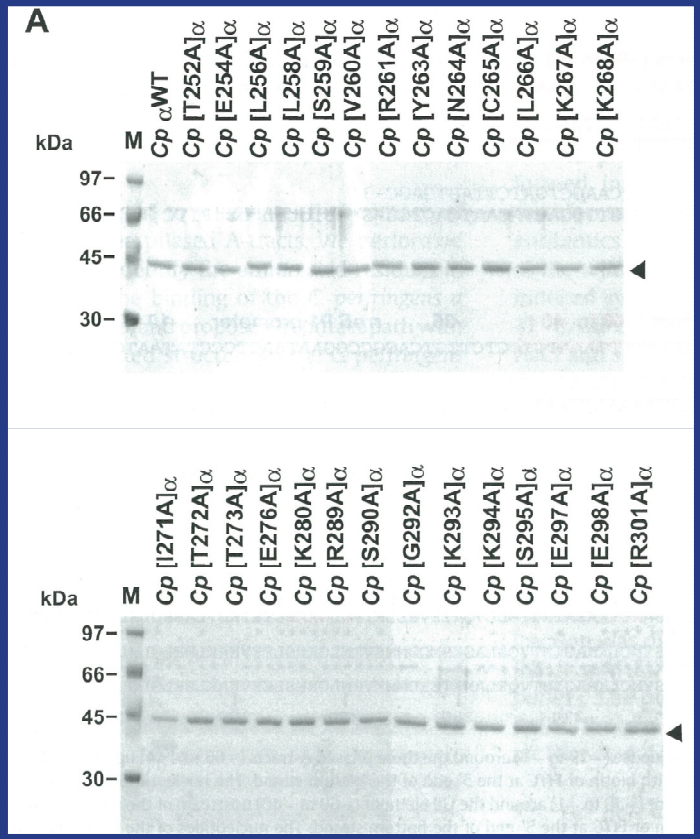


Red: the amino acid residues involved in binding of *E. coli* αCTD to UP element
Cyan: the amino acid residues in *Cp* αCTD substituted to alanine.

- To identify the amino acid residues involved in the binding to the phased A- tracts, 27 alanine substitutions in *Cp* αCTD were done.

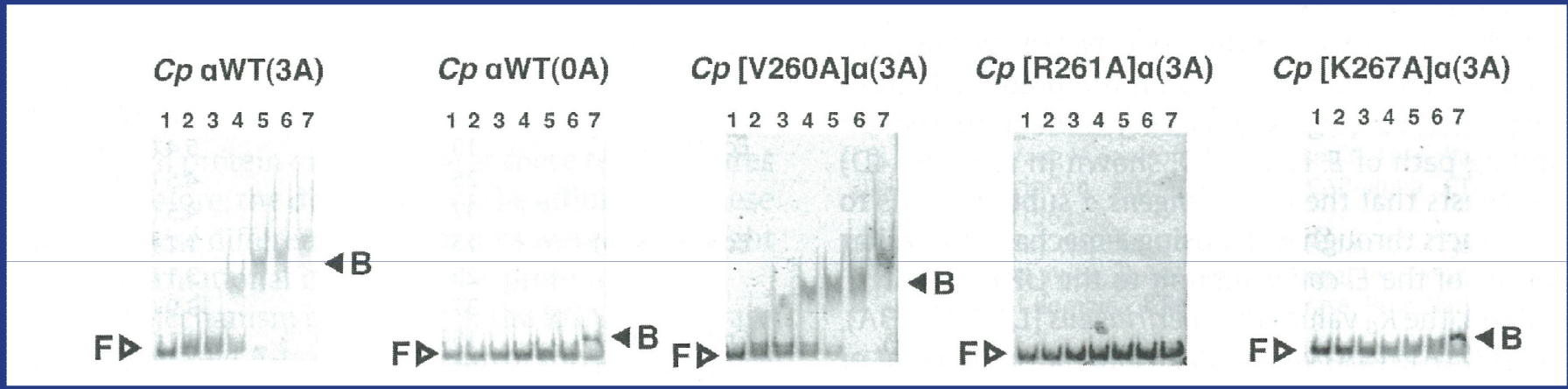
[Katayama, *et al*. *Anaerobe* **23**:62-69. 2013.]

Purified recombinant α subunits



- All α subunits were purified using a His_6-tag.

Gel shift assays with the mutated α subunits



- Five representative results were shown.

The results of gel shift assays and *K*d values estimated by SPR

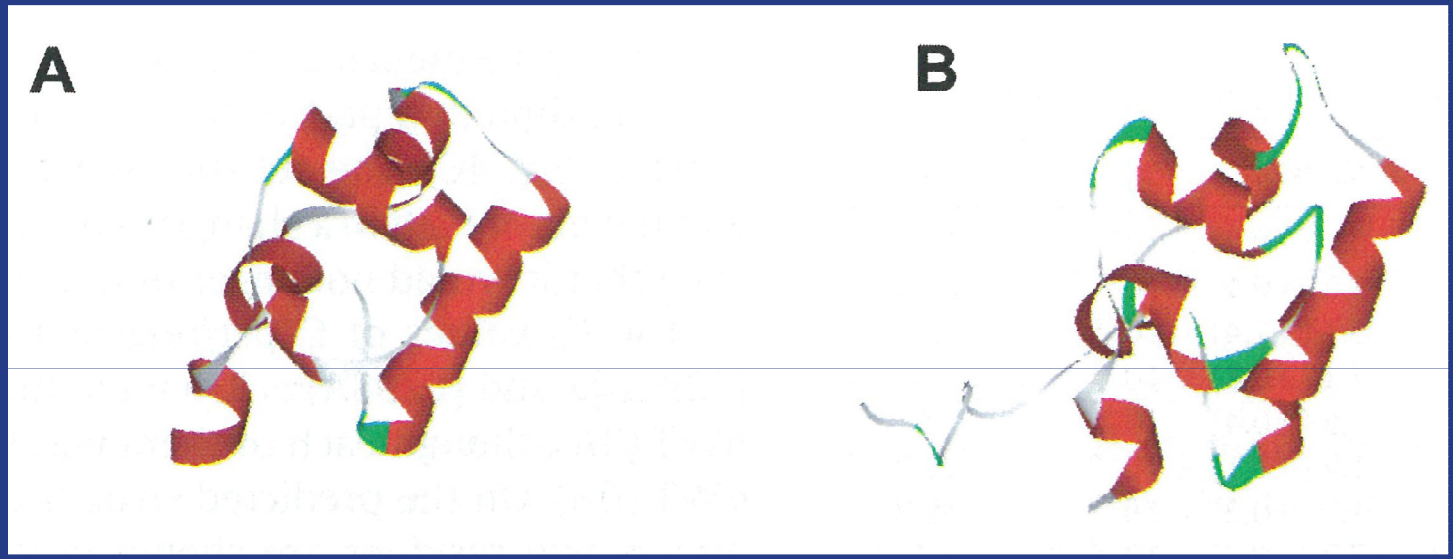
Table 1
Affinities of the *Cp* α subunits and mutated *Cp* α subunits to 3A or 0A DNA.

α Subunit (DNA)	Gel shift assay[a]	K_d (M)[b]	Ratio
αWT (3A)	++	$6.1 (\pm 0.3) \times 10^{-8}$	1.0
αWT (0A)	+	$1.5 (\pm 0.1) \times 10^{-6}$	24.3
[T252A]α (3A)	++	$6.1 (\pm 1.4) \times 10^{-8}$	1.0
[E254A]α (3A)	++	$7.1 (\pm 0.8) \times 10^{-8}$	1.2
[L256A]α (3A)	+	$1.5 (\pm 0.4) \times 10^{-7}$	2.5
[L258A]α (3A)	–	$1.4 (\pm 0.1) \times 10^{-6}$	22.8
[S259A]α (3A)	+	$1.2 (\pm 0.2) \times 10^{-6}$	18.8
[V260A]α (3A)	++	$3.2 (\pm 0.6) \times 10^{-8}$	0.5
[R261A]α (3A)	–	$2.1 (\pm 0.1) \times 10^{-4}$	342.6
[Y263A]α (3A)	++	$8.2 (\pm 1.4) \times 10^{-8}$	1.3
[N264A]α (3A)	–	$4.7 (\pm 0.3) \times 10^{-6}$	76.8
[C265A]α (3A)	++	$1.6 (\pm 0.4) \times 10^{-8}$	0.3
[L266A]α (3A)	–	$1.7 (\pm 0.1) \times 10^{-6}$	28.4
[K267A]α (3A)	+	$1.1 (\pm 0.1) \times 10^{-6}$	18.7
[R268A]α (3A)	++	$1.5 (\pm 0.1) \times 10^{-7}$	2.5
[I271A]α (3A)	–	$5.2 (\pm 0.2) \times 10^{-6}$	85.2
[N272A]α (3A)	++	$8.1 (\pm 0.1) \times 10^{-8}$	1.3
[T273A]α (3A)	+	$8.0 (\pm 0.9) \times 10^{-7}$	13.0
[E276A]α (3A)	++	$6.4 (\pm 0.8) \times 10^{-8}$	1.0
[K280A]α (3A)	+	$4.9 (\pm 0.3) \times 10^{-7}$	8.0
[R289A]α (3A)	++	$1.9 (\pm 0.5) \times 10^{-7}$	3.1
[N290A]α (3A)	++	$1.5 (\pm 0.1) \times 10^{-7}$	2.5
[G292A]α (3A)	–	$2.0 (\pm 0.1) \times 10^{-6}$	33.2
[K293A]α (3A)	++	$6.3 (\pm 0.0) \times 10^{-7}$	1.0
[K294A]α (3A)	–	$5.0 (\pm 0.8) \times 10^{-6}$	80.7
[S295A]α (3A)	–	$9.9 (\pm 1.6) \times 10^{-7}$	16.2
[E297A]α (3A)	+	ND[c]	ND
[E298A]α (3A)	+	$9.1 (\pm 1.0) \times 10^{-7}$	14.9
[R301A]α (3A)	++	$1.1 (\pm 0.0) \times 10^{-7}$	1.8

[a] The results of gel shift assays. ++: A gel shift was observed with dose of more than 3 μM protein. +: A gel shift was observed with dose of more than 5 μM protein. –: No gel shift was observed with dose of 5 μM protein.
[b] K_d values were determined by kinetic analyses of SPR. Values are the average of three independent experiments and the standard error is shown in parentheses.
[c] ND: Not determined.

- The results of gel shift assays were related to the dissociation constants (*K*d)

The predicted structure of *Cp* αCTD

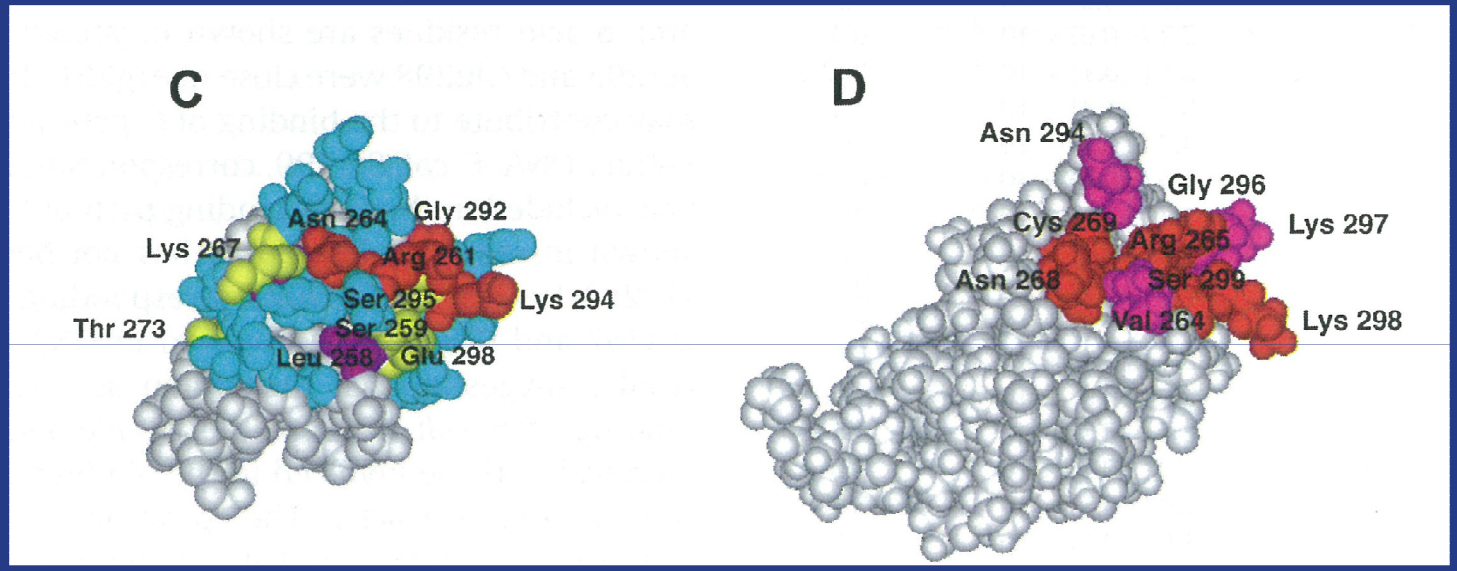


(A) *C. perfringens* αCTD

(B) *E. coli* αCTD

- The structure of *Cp* αCTD was predicted from that of *Bacillus subtilis* αCTD.

Mapping of amino acid residues substituted to alanine



(C) *C. perfringens* αCTD

Red : The values of *K*d increased more 30-folds than that of αWT.

Yellow : The values of *K*d increased more 8-folds than that of αWT.

Purple : important for the protein folding.

(D) *E. coli* αCTD

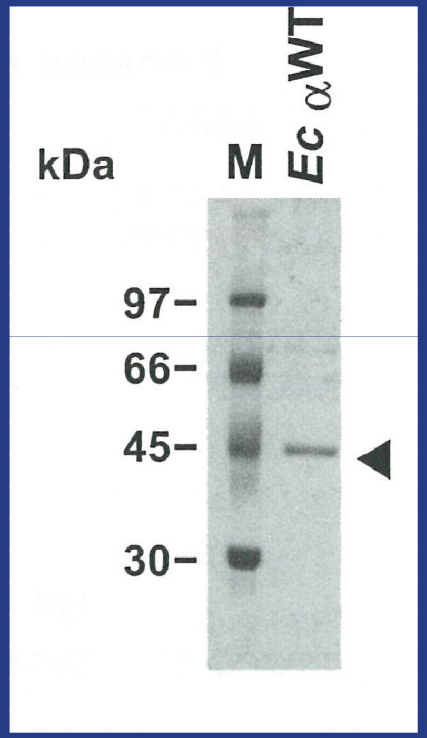
Red : The contact path between *E. coli* αCTD and the UPelement.

Pink: The amino acid residues involved in contact with the UP element.

[Gourse *et al*. *Mol Microbiol* **37**:687-695, 2000]

- Both contact paths were similar.

Affinities of the α subunits to DNA at various temperatures



α subunit (DNA)	Temperature (° C)	*Kd* (M)	Ratio
Cp αWT (3A)	15	$1.5 \pm 0.1 \times 10^{-8}$	1.0
	25	$6.1 \pm 0.3 \times 10^{-8}$	4.1
	37	$4.4 \pm 0.3 \times 10^{-7}$	30.8
Cp αWT (0A)	15	$6.5 \pm 0.5 \times 10^{-7}$	1.0
	25	$1.5 \pm 0.1 \times 10^{-6}$	2.3
	37	$9.8 \pm 0.6 \times 10^{-7}$	1.5
Ec αWT (UP)	15	$5.4 \pm 0.1 \times 10^{-9}$	1.0

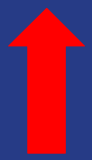
- The phased A-tracts was not simply a subset of UP element.

Summary

Three phased $A_{5\text{-}6}$-tracts (–66 to –40) lie upstream of *plc* gene promoter in *C. perfringens*

The αCTD of *C. perfringens* RNA polymerase

The minor grooves of the phased A-tracts

The phased A-tracts  The *plc* gene expression in a lowtemperature-dependent manner.

plc expression at room temperature may be important for *C. perfringens*

Animals, insects etc. on the ground

Death

C. perfringens, living in soil, happen to meet the dead body.

It is likely that they need much phopholipase C at room temperature to digest it.

Acknowledgements

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- Daisuke Nakamura M.S.
- Chiharu Tanaka M.S.

  [Okayama University of Science, Graduate School of Science, Japan]

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