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ORIGINAL ARTICLE

ComCED signal loop precisely regulates *nlmC* expression in *Streptococcus mutans*

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Abstract The ComED two-component signal transduction system (TCSTS) and the exogenous CSP can provoke the competence development and mutacin production of *Streptococcus mutans*. More binding fashions of ComE with DNA are the molecular basis for the ComED TCSTS to play pleiotropic roles. The interaction of *nlmC* and *comC* in regulation makes the expression of *nlmC* different from that of *nlmAB* and the ComE molecular feature is shown in the regulation pattern of *nlmC*. By electrophoretic mobility shift assay, ComE binding sites were discoveried in PnlmC314 and PnlmC419 for the first time. In the wild-type background, the promoter activity of *nlmC* is not only stronger than that of *nlmAB* but also rushes to a peak value of 3,116.0 MU at the point of 100 min in liquid culture with no supply of exogenous CSP; the data indicate that the regulation of *nlmC* expression is charged in an known coordination mechanism. In the mutant background, by monitoring the promoter activity of *nlmC* and *nlmAB*, it is proved that they are both controlled directly by the ComED TCSTS. But the surprising result is the deletion of *comC* to make *nlmC* promoter lose its activity completely. Based on those data, it is deduced that Pnlm419, PnlmC314 and PnlmC, which comprise the *nlmC* regulating region, coordinate to incite *nlmC* expression and to make *nlmC* expression different from that of *nlmAB* because of a synergistic effect, whereas they also could inhibit *comC* expression by blocking the RNA polymerase moving through the binding of ComE with the *comC* encoding region.

Keywords *Streptococcus mutans* · Mutacins · Electrophoretic mobility shift assay · Beta-Galactosidase

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Introduction

Streptococcus mutans is considered to be the primary pathogen causing dental caries. The known virulence factors of *S. mutans* include biofilm formation, acid production, competence development and bacteriocin (mutacin) synthesis (Li et al. 2001, 2002a, b; Cvitkovitch et al. 2003; van der Ploeg 2005; Kreth et al. 2005; Hale et al. 2005; Matsumoto-Nakano and Kuramitsu 2006; Kreth et al. 2007). The ability to produce mutacin is thought to play an important role in the establishment of the oral ecosystem. New interests are developing for mutacins because they are potential food preservatives and new effective antibiotics such as anticaries agents. Mutacins have two main groups, the lantibiotics and the non-lantibiotics. Lantibiotics are ribosomally synthesized and post-translationally modified peptides whereas non-lantibiotics consist of either one or two small unmodified peptides such as *nlmC* (Smu1738/SMU.1914C) and *nlmAB* (SMu0133/SMU.150, Smu0134/SMU.151) (Hale et al. 2005; Matsumoto-Nakano and Kuramitsu 2006). Recent research has demonstrated that the production of lantibiotic mutacin I is regulated by multiple inputs, while the production of non-lantibiotic mutacin IV and V (including *nlmAB* and *nlmC*) is controlled by the ComED two-component signal transduction system (ComED TCSTS) (Qi et al. 2001; van der Ploeg 2005; Kreth et al. 2005, 2006; Matsumoto-Nakano and Kuramitsu 2006; Kreth et al. 2007).

In *S. mutans*, the ComED TCSTS directly controls both non-lantibiotics bacteriocin production and competence for transformation, because ComE can recognize its specific sites in the regulation region of related operons to drive their transcription by the signal transduction of ComD, in response to the competence-stimulating peptide (CSP) encoding by *comC* (Li et al. 2002a, b; van der Ploeg 2005; Kreth et al. 2007; Hung et al. 2011a). Recent work has identified and biochemically characterized multiple genes with ComE binding sites in the vicinity of their promoter regions; these sites are classified into two types: in the operons of *comC*, *cslAB*, *nlmAB*, *immAB* and *vicRKX*, the binding sites are near to their initiator codons, while in the operons of *comED*, *comX* and *gtfB*, the binding sites are far from the codons (Hung et al. 2011a, b). Even more, one ComE binding site is approximately 1 kb from the translational initiator codon of *mbr* and near the end of the *gtfC* coding region (Hung 2009; Hung et al. 2011a, b). The polymorphism of ComE binding sites is not enough to incite the coordinated expression of both genes of competence development and mutacin production, while the unique feature of ComE protein is also important. The phosphorylated ComE proteins are easily induced to the formation of oligomers, ComE proteins and their oligomers can both bind to their recognition sites (Hung et al. 2011b), and the complex of ComE and DNA should offer more interacting behaviors with RNA polymerase to regulate gene expression, so the binding patterns of ComE with DNA allow ComE to undertake more functions. But today, we cannot precisely explain how ComED undertakes the tasks which are done, respectively, by two TCSTS of ComED and BlpRH in *Streptococcus pneumoniae* (Ween et al. 1999; Martin et al. 2000, 2006; Prudhomme et al. 2006) and how the ComCED loop is dampened to withstand the amplification effect because *comC* is not only a CSP structural gene but is also a member of a CSP-responsive and ComE-dependent autoregulatory loop. The *nlmC* regulation probably explains the molecular feature of ComE because the *nlmC* regulating region has complex ComE binding sites and its expression is also related to the regulation of *comC* gene.

The *nlmC* expression was controlled by the ComED signal and a conserved sequence in its promoter was pointed out to be a ComE recognized site, which was first reported in research on bacteriocin synthesis (van der Ploeg 2005). Then, Kreth et al. (2007) reported that an 11-bp direct repeat in the *nlmC-comC* intergenic region is necessary for ComE to perform both the activation of *nlmC* transcription and the repression of *comC* expression. Latterly, Hung et al. (2011b) demonstrated the equilibrium dissociation constant of ComE with its binding DNA and further characterized the binding sites in the *nlmC-comC* intergenic region. Taken together, the *nlmC* expression is involved to the *comC* regulation and displays ComE molecular mechanism. Gene *nlmA* and *nlmB* are organized in an operon and together encode a two-peptide nonlantibiotic mutacin IV bacteriocin, and its promoter (−1 to −195 bp) has 92.2 % identity to the *nlmC* promoter. The operon *nlmAB* expression has been particularly described by van der Ploeg (2005), and therefore it is used as a control in this study. The distinction of the *nlmC* expression pattern from that of *nlmAB* is mostly because operons *nlmC* and *comC* share a 266-bp intergenic region and are transcribed in the opposite direction from inside to outside (Fig. 1). The objective of this study is to determine the *nlmC* expression pattern and even to show the regulation mechanism of the ComCED loop.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used in this study, their relevant characteristics and genotypes are listed in Table 1. For plasmid proliferation and protein expression, *Escherichia coli* cells were used and maintained in LB medium at 37 °C with 50 μg/mL kanamicin, 100 μg/mL ampicillin or 1 mmol/L isopropyl beta-D-thiogalactopyranoside (IPTG) as needed. All *S. mutans* derivatives were grown in Todd-Hewitt broth medium with 0.3 % yeast extract. If antibiotic resistant clones were selected, the 1.2 % agar plates were supplemented with either 750 μg/mL kanamicin, 20 μg/mL erythromycin or both. All *S. mutans* strains were grown stationarily at 37 °C and the cells were transformed according to the protocol of Li et al. (2001). CSP was synthesized in Apeptide (http://www.apeptide.com).

DNA manipulation

Standard procedures were used for plasmid isolation, DNA digestion, ligation, cloning and polymerase chain reaction. Restriction enzymes and T4 DNA ligase were obtained from Fermentas. Taq DNA polymerase was obtained from Sangon (http://www.sangon.com/) and Invitrogen. PCR was performed with a Bio-Rad MyCycler thermocycler. All plasmids extracted from *E. coli* and DNA extracted from agarose gel were prepared, respectively, with the SanPrep Plasmid Preparation Kit (SK8192) and the SanPrep Gel Extraction Kit (SK8132) from Sangon (Table 2).

ComE extraction and electrophoretic mobility shift assay

The p41comE was constructed through inserting the *comE* encoding region into pET41a (+) between *NcoI* and *XhoI* sites and then it was transformed into BL21 (DE3) pLysS to get BL21/p41ComE. BL21/p41ComE cells were grown at 180 rpm until the cell density increased to the absorbance of 0.5~0.8 at 600 nm, then GST-ComE was overexpressed by induction with IPTG at the final concentration of 1 mmol/L at 25 °C for 4 h. GST-ComE fusion protein was extracted using GST Bind Purification Kit (Novagen 70794–3). The crude

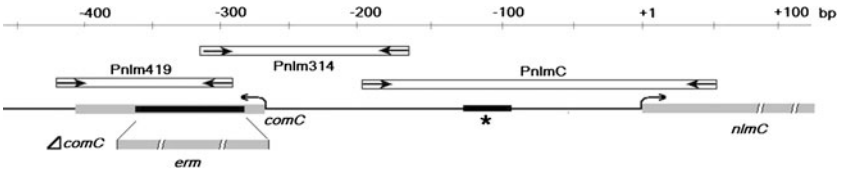

Fig. 1 Schematic diagram of *comC* and *nlmC* genomic region. *Gray box*: *comC*, *nlmC* and *erm* encoding region showed the directions of transcription with arrows. *Black box with star*: reported ComE binding site (van der Ploeg 2005; Kreth et al. 2007; Hung 2009; Hung et al. 2011a, b). *Long black box*: *comC* deletion region replaced by *erm* in ΔcomC. *Long blank box with arrows*: promoter PnlmC of *nlmC* and its upstream fragment PnlmC314 and PnlmC419

GST-ComE was dialysed against PBB buffer (pH7.5 20 mmol/L Tris-Cl, 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 0.05 % Tween 20 and 0.2 % Triton X-100). Approximately 10 μg GST-ComE was digested with enterokinase (Novagen 69066–3) in 40 μL and the mixture was stored at −20 °C.

EMSA was set up according to the method described by Jing et al. (2003), the reaction mixture was 20 μL pH7.5 buffer (20 mmol/L Tris-Cl, 25 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 0.05 % Tween 20, 0.2 % Triton X-100, 10 % glycerol, 50 μg BSA, 0→3.0 μg ComE and 100 ng DNA fragments). EMSA was run in 5 % (V/V) native polyacrylamide gel with Tris-Glycine-EDTA electrode buffer (25 mmol/L) at 4 °C.

Construction of *S. mutans* recombinant strains

The backbone vector for the construction of all reporter gene fusions is pLacZ which was generated by cloning the beta-*galactosidase* gene (*lacZ*) to shuttle vector pSF151. The fragments of LnlmC and LnlmAB including the promoters of *nlmC* and *nlmAB* were amplified by PCR with template of the chromosomal DNA of strain UA159 and were cloned into pLacZ to generate plasmid pA-LacZ and pC-LacZ.

Table 1 Bacterial strains and vectors used in this study

Strain and vector	Relevant characteristic	Reference
E. coli		
DH5α	*supE44 lacU169* (80*lacZ* M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 luxS*	(Hanahan 1983)
BL21(DE3)pLysS	F $^-$ *ompT gal hsdSB* (r_B^- m_B^-) *dcm lon* λDE3 pLysS	(Studier et al. 1990)
BL21/p41ComE	BL21 (DE3) pLysS habouring p41ComE to express fusion protein GST-ComE, Kan^R	This work
S. mutans		
WT	Wild-type strain UA159, Kan^S Erm^S	(Ajdic et al. 2002)
WT/pA-LacZ	WT with pA-lacZ by single cross insertion, Kan^R	This work
ΔcomX/pA-LacZ	As WT/pA-LacZ but *comX*::*erm*, Kan^R Erm^R	This work
ΔcomED/pA-LacZ	As WT/pA-LacZ but *comED*::*erm*, Kan^R Erm^R	This work
ΔcomC/pA-LacZ	As WT/pA-LacZ but *comC*::*erm*, Kan^R Erm^R	This work
WT/pC-LacZ	WT with pC-lacZ by single cross insertion, Kan^R	This work
ΔcomX/pC-LacZ	As WT/pC-LacZ but *comX*::*erm*, Kan^R Erm^R	This work
ΔcomED/pC-LacZ	As WT/pC-LacZ but *comED*::*erm*, Kan^R Erm^R	This work
ΔcomC/pC-LacZ	As WT/pC-LacZ but *comC*::*erm*, Kan^R Erm^R	This work
Vector		
pUCm-T	Cloning T-vector, Amp^R	Sangon (SK2211)
pET41a (+)	Expressing vector, Kan^R	Novagen (69337–3)
p41ComE	pET41a (+) insterted by *comE* to express fusion protein GST-ComE, Kan^R	This work
pSF151	Shuttle vector, Kan^R	(Tao et al. 1992)
pLacZ	pSF151 habouring gene *lacZ*, Kan^R	This work
pA-LacZ	pLacZ with the promoter of *nlmAB* to drive *lacZ*, Kan^R	This work
pC-LacZ	pLacZ with the promoter of *nlmC* to drive *lacZ*, Kan^R	This work

Table 2 PCR primers and the targets of amplified fragments

Primer	Primer sequence (5′→3′)	Sequence targeted
ComE-F	tcCCATGGTAATGATTTCTATTTTTGTATTGGAAG	*comE* for protein expression
ComE-R	tgCTCGAGTCATTCATTTTGCTCTCCTTTGAT	
PnlmA-F	AAATTAGCTGGTAATGATAGTT	*nlmAB* promoter (PnlmAB) (−163 to −43)
PnlmA-R	GCAACCAACATCTTTAGTATAA	
Nest-nlmC-F	AAATCCAGCCTTGTTTTATTCC	Upstream region of *nlmC* used in nest PCR for the amplification of *nlmC* regulating region (−444 to+103)
Nest-nlmC-R	GAGCAATACCTGCTGCACAA	
PnlmC-F	AAAGTAATATTTTCCTTAGGAC	*nlmC* promoter (PnlmC) (−197 to+51)
PnlmC-R	AAGTGCTTCATTATCCATTA	
PnlmC314-F	GTCTTAATTTCTTTAAAGTC	Upstream region of *nlmC* promoter (PnlmC314) (−314 to −166)
PnlmC314-R	TACATTATGTGTCCTAAGGA	
PnlmC419-F	GTTAGCCTATCTTATTTTCC	Upstream region of *nlmC* promoter (PnlmC419) (−419 to −295)
PnlmC419-R	GACTTTAAAGAAATTAAGAC	
Erm-H3	gaAAGCTTCCGGGCCCAAAATTTGTTTGAT	*erythromycin* (*erm*) for pLacZ construction
Erm-XO	aaCTCGAGTCGGCAGCGACTCATAGAAT	
upComED-KN	GAACATAATTTACAGCGGTTCATA	*comE* and its upstream region (−231 to+371) for *comED* mutant construction
upComED-H3	ggAAGCTTCAATGCGGTGGGAGAACT	
downComED-XO	gaCTCGAGTTAGGCGGGCAATCATATTC	*comD* and its downstream region (+ 289 to+1069) for *comED* mutant construction
downComED-BH	AGCAGCCTCAATGGCATTAT	
upComC-KN	ATCTGAACAAGCAGGGGAGA	*comC* and its upstream region (−752 to+13) for *comC* mutant construction
upComC-H3	gcAAGCTTGTGTTTTTTTCATTTTATATCTCC	
downComC-XO	taCTCGAGTCCGGCTGTTTAACAGAAGTT	*comC* and its downstream region (+ 98 to+940) for *comC* mutant construction
downComC-BH	GGCACAAAAGGAAGCTCAGA	
upComX-KN	TTAGTTTGTAATGCGAGGTG	*comX* and its upstream region for *comX* mutant construction
upComX-H3	gcAAGCTTAACGACGTTTCTGACTTTCT	
downComX-XO	tgCTCGAGGAAGTCGGTGAGATTGAACA	*comX* and its downstream region for *comX* mutant construction
downComX-BH	GACCAAGATTGATTTTAGCC	
LacZ-XF	gaTCTAGAGCCACACAGGAAACAGCTATGAC	Beta-*Galactosidase* (*lacZ*) used as a reporter in the analysis of promoter activity
LacZ-SR	aaGTCGACGAAGTAGGCTCCCATGATAAA	
LnlmA-BF	gcGGATCCGTATCGGAAGAATTATCTGG	*nlmA* upstream region (−741 to+56) used in pA-LacZ construction
LnlmA-XR	atTCTAGAGGTTGAAAGTGTTTGGCTGT	
LnlmC-BF	aaGGATCCTTTTATCTTCTCATCCACGAC	*nlmC* upstream region (−523 to+122) used in pC-LacZ construction
LnlmC-XR	agTCTAGAGTAACCTTGCCCAGCACCTA	

Underlined letters are restriction site sequences. PCR procedures were set up according to the manuals of Taq polymerase (Invitrogen 10966–018 or Sangon BBI B0089) and performed for 25 cycles. *Erythromycin* cassettes were recovered by PCR with upCom and downCom primers after upCom-*erm*-downCom were ligated together

Then, the plasmids were transformed into *S. mutans* strain UA159 to get WT/pA-lacZ and WT/pC-lacZ via single crossover recombination and the transformants were confirmed by PCR and the activity analysis of reporter gene.

The mutants related to ComED TCSTS were constructed according to the protocol of van der Ploeg (2005). The upCom-*erm*-downCom fragments of *erm* cassettes were tranformed to WT/pA-lacZ and WT/pC-lacZ to get the defective strains of *comX*, *comC* and *comED* by double crossover recombination, and the transformants were confirmed by PCR.

Characterization of *nlmC* and *nlmAB* promoters by beta-galactosidase analysis

The beta-galactosidase (LacZ) activities were tested with the method described by Peruzzi et al. (1998). The 1:20 dilution of overnight culture was incubate at 37 °C in 3 h and then the cells were harvested at 10-min intervals to test beta-galactosidase activity. To test the effect of the ComED TCSTS on *nlmC* and *nlmAB* expression, the samples of WT and each mutant strain were collected after the 1:20 dilution of overnight culture was incubated at 37 °C for 2 h. CSP was added to a final concentration of 0.8 μg/mL if needed. The beta-galactosidase activity is calculated according to the formula: Miller Units=[10,00×A_{420}/reaction time (min)]×OD_{675}, in which the beta-galactosidase activity was determined by the absorbance at 420 nm in the color reaction of O-nitrophenyl-beta-D-galactopyranoside (ONPG) and the cell density of each smaple was determined by the absorbance at 675 nm (Peruzzi et al. 1998).

Results

Binding reactions of ComE with 4 selected DNA fragments are specific

The EMSA results indicate that all the binding reactions of ComE with PnlmAB, PnlmC, PnlmC314 or PnlmC419 are specific, because in each reaction the main retarded DNA bands are monitored (Fig. 2). For the binding reactions of ComE with PnlmAB, the EMSAs show 3 main retarded DNA bands (Fig. 2a, b1 to b3), whereas in the reactions of ComE with PnlmC, PnlmC314 or PnlmC419, each EMSA displays 2 main retarded DNA bands (Fig. 2b–d, b1 and b2).

Effect of exogenous CSP on *nlmC* and *nlmAB* expression

In the wild-type background, if not supplying exogeous CSP, the expression of *nlmC* is outstandingly distinctive from that of *nlmAB* in the continuous liquid culture (Fig. 3, dashed line). First, the activity of *nlmAB* promoter rises slowly at a low level from approximate 100 to 200 MU in 3 h (Fig. 3, dashed line with square symbols), while at 70 min, the promoter activity of *nlmC* runs at approximately the 400 MU level, but from 70 to 100 min its activity rushs sharply up to 3,116.0 MU in 30 min and then decreases quickly to about 1,700 MU in about 60 min (Fig. 3, dashed line with triangle symbols), the results indicating that the promoter activity of *nlmC* did not change in a linear manner but an unknown coordination mechanism. But if supplying exogenous CSP, the activities of *nlmC* and *nlmAB* promoters change in the same tendency except that the activity of *nlmC* promoter is stronger than that of the *nlmAB*'s at the same point of the growth period (Fig. 3, solid line).

Gene *nlmC* expression on the background of mutant *comX*, *comED* and *comC*

Because *comX* is one of the downstream genes controlled by ComE, in mutant ΔcomX or WT backgrounds, the corresponding promoter activity changes almost in the same way, respectively (Table 3), whereas the activity of *nlmC* promoter is obviously stronger than that of *nlmAB* promoter no

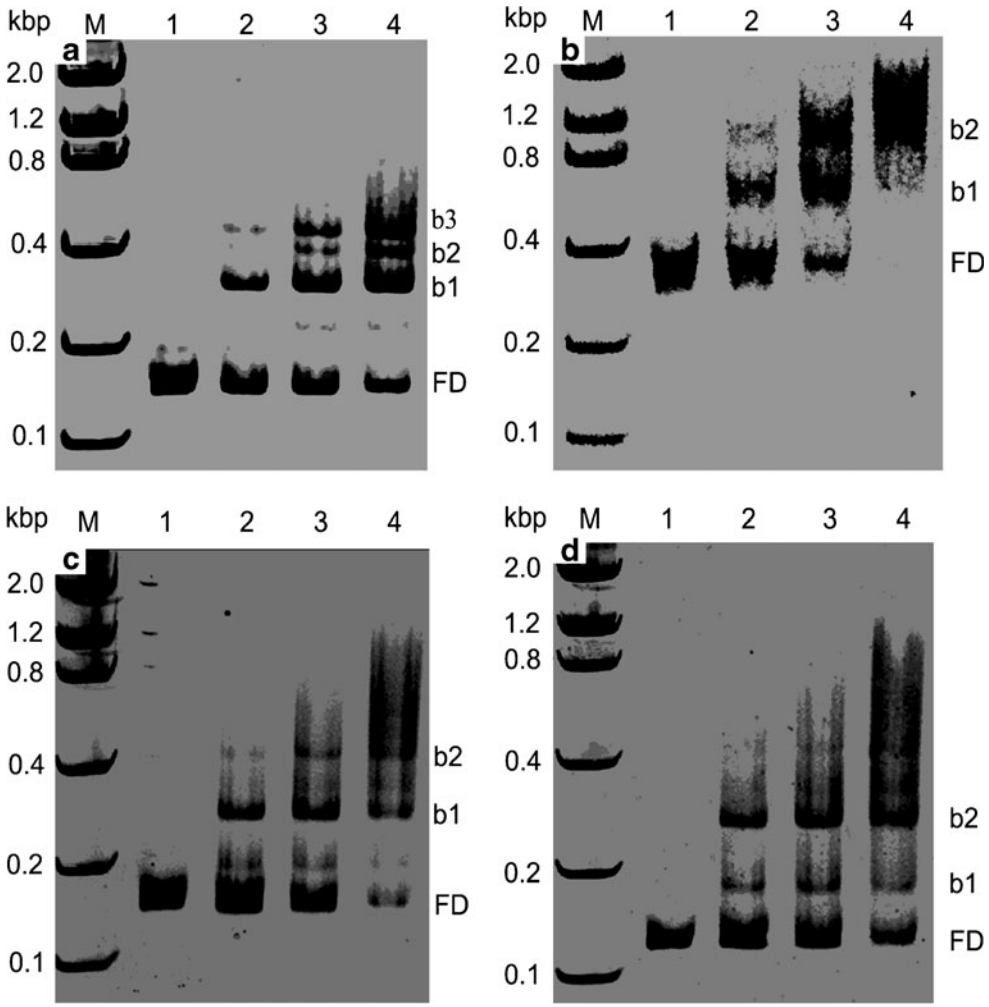

Fig. 2 Electrophoretic mobility shift assay of ComE with 4 selected DNA fragments. **a**, **b**, **c**, **d**: EMSAs of ComE with PnlmAB, PnlmC, PnlmC314, or PnlmC419, respectively. *M*: DNA ladder (Invitrogen 10068–013). *Lanes 1–4*: ComE concentration gradient of 0, 326, 652, and 1,305 ng per 20 μL volume, respectively. *FD*: Free DNA. *b1*, *b2*, *b3*: main retarded DNA

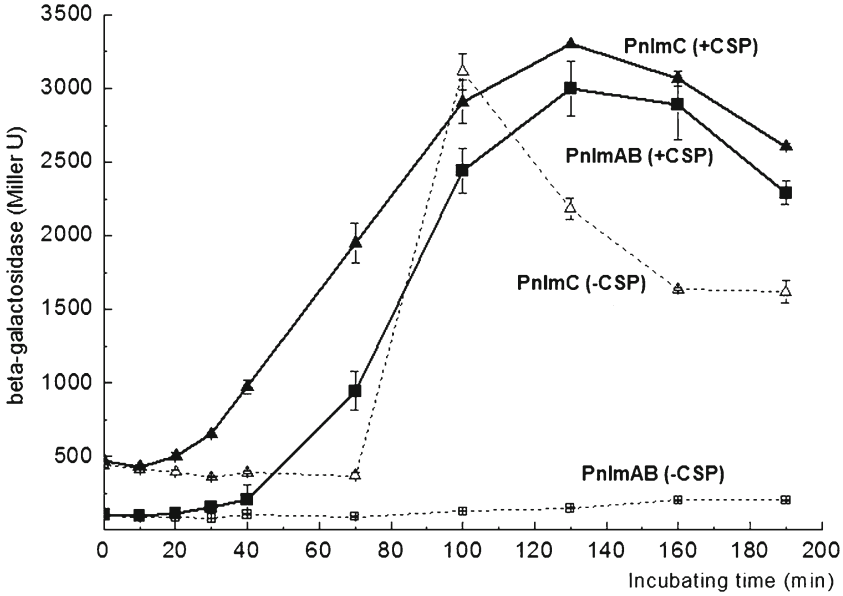

Fig. 3 Effect of exogenous CSP on *nlmC* and *nlmAB* expression. Expression of *nlmC* and *nlmAB* is detected by beta-galactosidase activity in the cassette of promoter–*lacZ* which is integrated in the genome of WT/pA-LacZ or WT/pC-LacZ. *Dashed line*: liquid medium without exogenous CSP. *Solid line*: liquid medium supplying with 0.8 μg/mL CSP. *Square symbols* (■, □): WT/pA-LacZ. *Triangle symbols* (▲, △): WT/pC-LacZ

matter whether in WT or in ΔcomX; if not supplying exogeous CSP, the activity of *nlmC* promoter is 4- to 6-fold stronger than that of *nlmAB* promoter, whereas if treating with exogenous CSP, it is about 1.4-fold stronger (Table 3). The ComED TCSTS is the only signal transmission to start up the expression of *nlmC* and *nlmAB* responding to exogenous CSP, because in ΔcomED the promoter activities not only decrease to the lowest level of 48.5 MU or 57.8 MU but are also no longer incited by exogenous CSP (Table 3). In mutant ΔcomC, the *nlmAB* promoter activity is almost made up to the normal level as in WT or mutant ΔcomX by supplying exogenous CSP, but the *nlmC* promoter activity does not recover at all (Table 3). It is truly surprising that an intact *comC* is necessary for the *nlmC* promoter to be induced by exogenous CSP.

Table 3 Effect of mutant *comX*, *comED* and *comC* on *nlmC* expression

Strain[a]	Treatment[b]	PnlmAB (Miller Unit)[c]	PnlmC (Miller Unit)[c]
WT	− CSP	247.0±6.0	988.8±47.8
	+ CSP	2307.1±84.1	3155.9±99.3
ΔcomX	− CSP	174.8±1.7	1063.4±125.2
	+ CSP	2261.3±34.3	3273.3±156.1
ΔcomED	− CSP	48.5±1	57.8±10.5
	+ CSP	55.8±5.2	40.8±14.6
ΔcomC	− CSP	153.2±8.6	19.3±1.1
	+ CSP	2231.5±116.3	22.0±1.8

The beta-galactosidase activity was calculated according to the formula: Miller Units=[10,00×A_{420}/reaction time (min)]×OD_{675} and the absorbance of A_{420} and OD_{675} were ONPG color reaction of beta-Galactosidase and the cell density, respectively, defined in "Materials and methods"

[a] Background of *S. mutans* strains discribled in Table 1. *WT*, *ΔcomX*, *ΔcomED*, *ΔcomC*: wild-type and deletion mutants of *comX*, *comED* or *comC* gene

[b] *− CSP*: not supplying exogenous CSP as control in medium, *+ CSP*: supplying with 0.8 μg/mL CSP in medium

[c] Different background strains harboring *nlmAB* promoter-*lacZ* (*PnlmAB*) or *nlmC* promoter-*lacZ* (*PnlmC*)

Dicussion

In *S. mutans*, the exogenous CSP and the ComED TCSTS are able to promote competence development and mutacins synthesis (Li et al. 2001, 2002a, b; Kreth et al. 2005; van der Ploeg 2005; Kreth et al. 2006, 2007). To confirm ComE binding sites and to discover the molecular properties of ComE proteins are the key to rebuilding the regulation network of ComED TCSTS. Hung and his colleagues not only scanned the whole genome to discover ComE binding sites which they proved to be 'TCBTAAAYSGT' of a 11-bp single site consensus sequence but also proved that the phosphorylation state of ComE affects the formation of ComE oligomers not to change its affinity with DNA (Hung 2009; Hung et al. 2011a, b); this conclusion confirms that ComE can bear more tasks. Here, it is the first time that PnlmC314 and PnlmC419 have been proved to harbor ComE bindng sites, even more in mutant ΔcomC, in which the 85-bp *comC* encoding region overlapping with PnlmC314 and PnlmC419 is deleted; *nlmC* expression was almost a complete failure (Fig. 1; Table 3), so we believe the deleted fragment must be an important cis-element which is recognized by ComE proteins or an unknown protein which controls the expression of *nlmC*. Other researchers have

demostrated that the *nlmC-comC* intergenic region overlapping with the PnlmC plays roles to excite *nlmC* expression and repress *comC* expression, released a head-to-tail fashion to explain their regulation mechanism (Hung 2009; Hung et al. 2011b). In this work, the promoter activities displayed in WT/pC-lacZ from WT/pA-lacZ were quite different (Fig. 2a, Table 3), which must be brought into being by the upstream sequences of their promoters not their promoter sequences because of the 92.2 % identity in their −1 to −195 bp promoter region. The binding action of ComE with PnlmC314 or PnlmC419 should increase the interaction between PnlmC and ComE to make the *nlmC* promoter become stronger as well as change the coordinated pattern in the middle of the liquid culture period (Fig. 3). Although the interaction in the bindings of ComE with PnlmC, Pnlm314 and PnlmC419 is not confirmed, we hypothesize that the 3 fragments are recognized coordinately by ComE proteins to precisely perform *nlmC* expression control and make the *nlmC* expression pattern different from that of *nlmAB*.

On mutant analysis, the ComED TCSTS is believed to be the only pathway to start up the transcription of *nlmC* and *nlmAB* to respond to the excogenous CSP stimulation (Table 3); the same results have also been shown by other researchers (van der Ploeg 2005). To regulate mutacins production is the primary role of ComED because BlpRH of *S. pneumoniae* is a ComED homologue which controls mutacins sythesis independently (Qi et al. 2005; Martin et al. 2006; Allan et al. 2007). By analogy with this model, the regulation of ComED to *nlmAB* expression should share the similar molecular mechanism. Surprisingly, the *comC* encoding region is necessary for exogenous CSP to activite *nlmC* expression (Table 3). In mutant ΔcomC, *erm* gene is drived by *comC* promoter, and because the mutant ΔcomC cells can grow normally in the medium supplying erythromycin, the promoter of *comC* must work very well, so we deduce that Pnlm314 and PnlmC419 (*comC* encoding region) act as a cis-element to provoke the gene transcription in one direction and the deleted 85-bp fragment must be recognized by ComE protein or an unknown repressor. The ComE protein has been believed to be a bifunctional protein, and a head-to-tail fashion was used to answer for ComE molecular function in the cooperating control of *nlmC* and *comC* transcription (Kreth et al. 2007; Hung 2009; Hung et al. 2011a, b). But this model does not readily explain that ComE could also incite the expression of the operon *comED*, *comX* or *cslAB* in the same manner, so we believe that the ComE–DNA complex normally activates its effectors transcription like *nlmC* or *nlmAB* in one direction because of the polarity and asymmetry of ComE protein and its binding sites, but the complex of ComE with PnlmC314 or PnlmC419 inhibits the transcription of the *comC* gene by preventing RNA polymerase movement. Certainly, the signal loop of ComCED is dampened by the restraint of *comC* expression.

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