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Phase-variation of pyelonephritis-associated pili in *Escherichia coli*: evidence for transcriptional regulation

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The regulation of pyelonephritis-associated pili (*pap*) pilin gene transcription has been examined using two operons (*pap*-17 and *pap*-21) isolated from the pyelonephritogenic *Escherichia coli* strain C1212. DNA sequence analysis and *E.coli* minicell analysis were used to map two genes (*pap*B and *pap*I) within the pilin regulatory regions of both *pap*-17 and *pap*-21, and the protein products of these genes were identified. Pilin transcription, initiated at the *pap*BA promoter, was monitored by constructing single copy operon fusions with *lac*ZYA in *E.coli* K-12. Inoculation of *E.coli* (*pap'*−*lac*) strains onto solid M9 minimal medium containing glycerol and the Lac indicator X-gal (M9-Glycerol) yielded both Lac^+ and Lac^- colony phenotypes. The Lac^+ ('phase on') and Lac^- ('phase off') phenotypes were heritable since reinoculation of M9-Glycerol with bacteria picked from Lac^+ colonies gave rise to a much higher fraction of Lac^+ colonies than reinoculation of M9-Glycerol with bacteria picked from Lac^- colonies. Measurement of phase transition rates for *E.coli* (pap_{17}'−*lac*) inoculated onto M9-Glycerol showed that the $Lac^- \rightarrow Lac^+$ transition frequency (1.57×10^{-4}/cell/generation) was reduced 35-fold when cells were inoculated onto minimal medium containing glucose (M9-Glucose). However, the $Lac^+ \rightarrow Lac^-$ transition frequency obtained using M9-Glycerol (2.60×10^{-2}/cell/generation) was 1.4-fold lower compared to results obtained with M9-Glucose. In contrast, lowering the incubation temperature of *E.coli* (pap_{17}'−*lac*) cultures from 37°C to 23°C caused all cells to shift to the Lac^- state. Together, our results strongly indicate that *pap* pili phase-variation is transcriptionally regulated and show that phase-variation is responsive to changes in the bacterial environment.

Key words: gene fusion/*pap*/phase-variation/transcriptional regulation/urinary tract infections

Introduction

Most *Escherichia coli* that cause upper urinary tract infections (pyelonephritis) contain the pyelonephritis-associated pili (*pap*) gene cluster (O'Hanley *et al.*, 1985). The *pap* gene cluster consists of at least nine genes, designated as *pap*A→*pap*I (Normark *et al.*, 1983; Norgren *et al.*, 1984), that are involved in the synthesis and assembly of a pilus complex at the bacterial cell surface (Lund *et al.*, 1988). This complex consists of a pilus fiber composed of pilin monomers encoded by *pap*A and at least three additional proteins encoded by *pap*E, *pap*F and *pap*G which appear to be located at the pilus tip (Lindberg *et al.*, 1984; Lindberg *et al.*, 1987). The PapG protein appears to function as the adhesin that binds to specific receptors on target host epithelial cells (Lund *et al.*, 1987).

The pyelonephritogenic *E.coli* strain C1212 contains two complete *pap* gene clusters, each encoding antigenically distinct pilin monomers designated as pilin-17 and pilin-21 (Low *et al.*, 1987). Each *pap* gene cluster (*pap*-17 and *pap*-21 respectively) also encodes an adhesin that binds to terminal digalactoside residues (Low *et al.*, 1984). Using immunoelectron microscopy we showed that most bacterial cells (84%) within a colony expressed pilin-21 but only about 5% of cells expressed pilin-17. Pilin expression appeared to be an 'all or none' phenomenon; bacteria either expressed cell surface pili or expression was not detected. Based on these results we concluded that *pap* pilin expression was under the control of a phase-variation mechanism; phase-variation being defined here as the oscillation of a cell between alternate expression states.

In this study we constructed single copy operon fusions that placed the *lac*ZYA operon under the control of the promoter for pilin-17 and pilin-21. Results obtained using these fusions strongly indicate that *pap* pili phase-variation is transcriptionally regulated. In addition, our results show that this phase-variation is heritable and is responsive to changes in both the carbon source used for growth and incubation temperature.

Results

DNA sequence analysis of the pap pilin regulatory region

DNA sequence analysis of the pilin genes and upstream regulatory sequences of *pap*-21 and *pap*-17 was performed as described in Materials and methods. *Pap*-17 shared more than 97% sequence similarity to *pap*-21 (Figure 1). Computer analysis of the *pap* DNA sequences shown in Figure 1 indicated that there were two open reading frames upstream from the *pap*A (pilin) gene. These open reading frames are preceded by potential promoters and ribosome binding sites as shown in Figure 1, and correspond both in sequence and position to those of *pap*I and *pap*B as shown by M.Baga *et al.* (1985) using a similar, but not identical, *pap* DNA sequence. Based on the DNA sequence, *pap*I and *pap*B appear to be transcribed from divergent promoters. *Pap*A seems not to have a separate promoter region and evidence indicates that it is co-transcribed with *pap*B from a single promoter designated as the *pap*BA promoter (Baga *et al.*, 1985, 1988). There is also a possible CAP−cAMP binding site located between the *pap*I and *pap*B

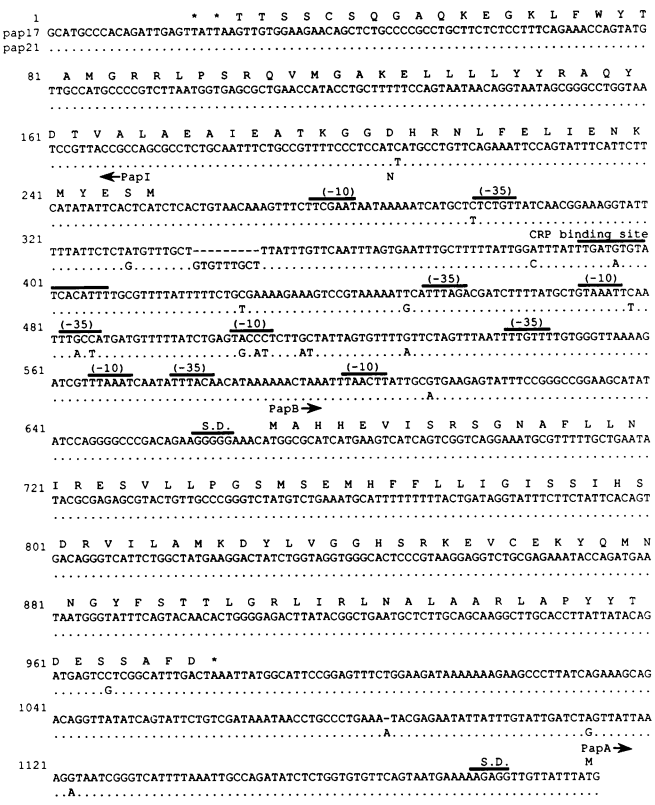

Fig. 1. DNA sequence analysis of the *pap*-17 and *pap*-21 regulatory regions. The DNA sequence of the pilin regulatory regions of *pap*-17 and *pap*-21 was determined by the method of Sanger *et al.* (1977) and described in Materials and methods. The *pap*-17 DNA sequence is presented on the top line. The *pap*-21 DNA sequence is presented below, with dots indicating the same nucleotide as shown on the line above. The absence of nucleotide base-pairs in one DNA sequence is shown by dashes and the one-letter amino acid code is given above the codons of the predicted open reading frames. The *Pap*A DNA sequence for both *pap*-17 and *pap*-21 will be presented elsewhere (Blyn, Braaten, Wals, Low and O'Hanley, in preparation).

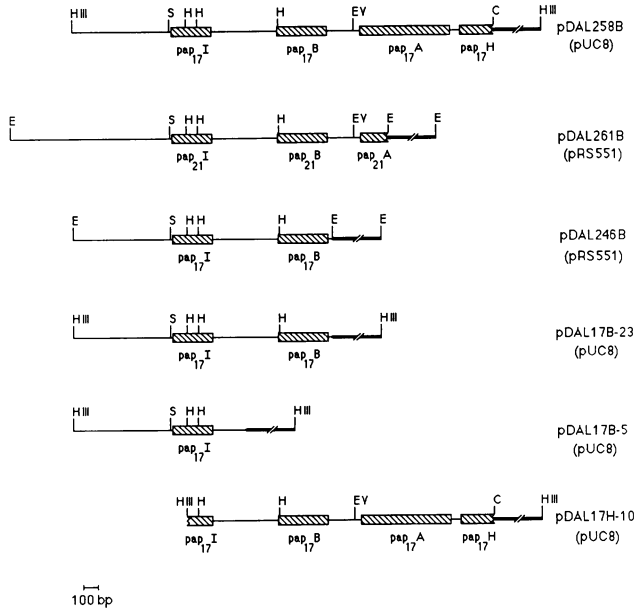

Fig. 2. Plasmid deletion constructs containing *pap*-17 and *pap*-21 DNA sequences. Hatched bars represent open reading frames which correspond to the gene shown below each bar [determined by deletion mapping (see text)]. Broken hatched bars indicate interrupted reading frames. The thick black line represents the vector used in the construct (pUC8 = 2.6 kb, pRS551 = 12.5 kb). The vectors used for each plasmid construct are shown in parentheses. Abbreviations used are: C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hha*I; HIII, *Hin*dIII; S, *Sph*I.

genes. The small number of differences between the *pap*-17 and the *pap*-21 sequences were concentrated in the regulatory region located between *pap*I and *pap*B.

Analysis of proteins encoded by pap-17 and pap-21

The proteins encoded by *pap*-17 and *pap*-21 were analyzed using *E.coli* minicells (Dougan and Kehoe, 1984). Analysis was facilitated by constructing plasmids that contained portions of *pap*-17 and *pap*-21 (Figure 2 and Materials and methods). These plasmid constructs were transformed into the *E.coli* minicell strain ORN103 (Orndorff *et al.*, 1985). Minicells were isolated by sucrose gradient centrifugation, and [^{35}S]methionine labeled proteins were resolved by electrophoresis on modified SDS−PAGE gels which allowed the resolution of low mol. wt proteins (Giulian *et al.*, 1985). Our results indicated that the *pap*-17 and *pap*-21 pilin regulatory regions shown in Figure 1 encode two proteins of ~10 and 12 kd respectively (Figure 3A, lanes 4 and 5; Figure 3B, lanes 2 and 3). In addition, a 16 kd protein was encoded by *pap*-17 DNA sequences containing a portion of the pap_{17}H gene (Figure 2, plasmids pDAL17H-10 and pDAL258B and Figure 3A, lanes 3 and 4). Based on DNA sequence analysis (our unpublished data), it seems likely that the 16 kd protein is encoded by an out of frame fusion of a portion of the pap_{17}H gene to *lac*Z. The Pap_{17}A (pilin) protein, encoded by plasmids pDAL258B and pDAL17H-10 [as determined by both protein and DNA sequence analysis; (Blyn *et al.*, manuscript in preparation)], was not visualized (Figure 3a, lanes 3 and 4). This might have been due to the instability of pilin in the absence of the PapD protein, which appears to be involved in pilin assembly (Norgren *et al.*, 1987).

To confirm that the 10 and 12 kd proteins visualized by SDS−PAGE analysis were encoded by the open reading frames identified as *pap*I and *pap*B (Figure 1), *E.coli* minicell analysis using deletion subclones was performed (Figure 3A). Plasmid pDAL17B-5, which contains only the pap_{17}I open reading frame (ORF), yielded a single protein of apparent mol. wt 10 kd when transformed into *E.coli* minicells. Plasmid pDAL17H-10, containing the complete pap_{17}B and pap_{17}A ORFs and partial pap_{17}I and pap_{17}H ORFs, yielded 12 and 16 kd proteins (discussed above) but lacked the 10 kd protein after transformation into *E.coli* minicells. Together, these results indicated that the pap_{17}I ORF encoded the 10 kd protein. Plasmid pDAL17B-23, containing both the pap_{17}I and pap_{17}B ORFs, yielded 10 and 12 kd proteins in *E.coli* minicells. These results, together with results using plasmid pDAL17B-5, indicated that the pap_{17}B ORF encoded the 12 kd protein. Furthermore, the mol. wts obtained for Pap_{17}I and Pap_{17}B by DNA sequence analysis (8.6 and 11.6 kd, respectively) corresponded well with the apparent mol. wts obtained by SDS−PAGE analysis. Analysis of *E.coli* minicells transformed with pDAL261B, which contains ORFs encoding Pap_{21}I and Pap_{21}B, showed that two proteins of mol. wts similar to

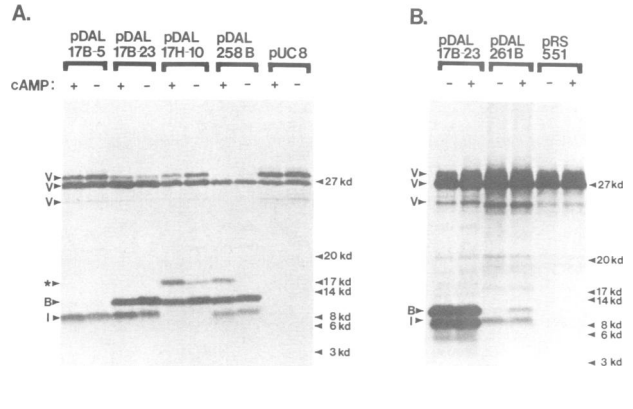

Fig. 3. Analysis of proteins encoded by plasmid constructs using *E.coli* minicell analysis. *E.coli* minicells containing the plasmids listed below were isolated and incubated with a mixture of [^{35}S]cysteine and [^{35}S]methionine (*Trans*-^{35}S-label, ICN) as described in Materials and methods. ^{35}S-labeled proteins were separated on a SDS−PAGE gel system designed for low mol. wt proteins (Giulian *et al.*, 1985) and visualized by fluorography as described above. **Panels A** and **B** show results obtained using the plasmids listed above each set of lanes. '+' indicates that cAMP (3 mM) was added to minicell preparations; '−' indicates no addition of cAMP; 'V' shows the locations of vector-encoded proteins; 'B' and 'I' show the locations of the *pap*B and *pap*I proteins respectively. The asterisk shows the location of a putative pap_{17}H−*lac*Z fusion protein (see text).

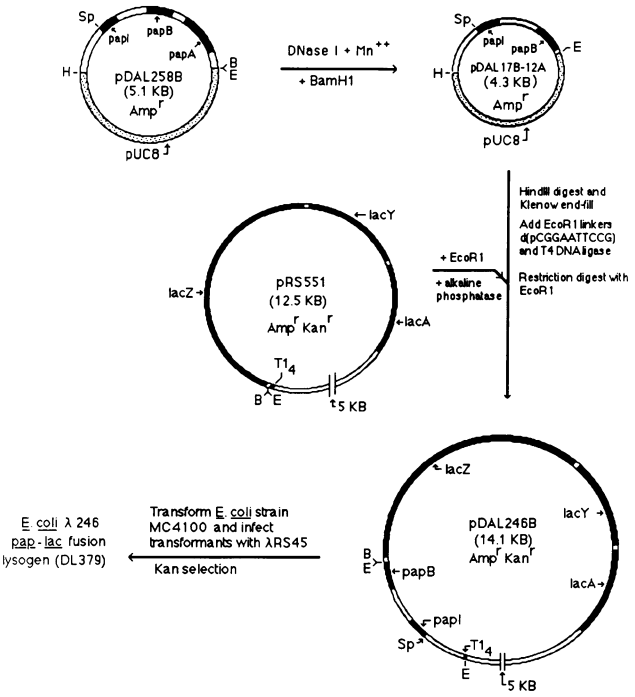

Fig. 4. Construction of *E.coli* strain DL379: a single copy pap_{17}′−*lac* fusion lysogen.

Pap_{17}I and Pap_{17}B were produced (Figure 3B). The visualization of the *pap*I and *pap*B gene products was remarkable in that they had not previously been resolved from each other due to their low mol. wts (Norgren *et al.*, 1984; Low *et al.*, 1987). cAMP (3 mM final concentration) was added to all of the *E.coli* minicell preparations because we noted that pRS551 plasmid constructs (Figure 3B and unpublished data) did not produce easily detectable amounts of PapB in the absence of cAMP. Levels of the putative PapH fusion protein were also increased by cAMP addition (Figure 3A, pDAL17H-10 and pDAL258B). However, as shown in Figure 3, consistent results were not obtained using pUC8 derivatives. pUC8 plasmid derivatives produced higher levels of PapI and PapB than pRS551 derivatives (Figure 3B). Because of the inconsistencies noted above, these results cannot be used to determine the role(s) of cAMP on regulation of *pap*B and *pap*I gene expression.

Fusion of the pap pilin promoter to the lac operon

Previous results has shown that expression of pilin from the *pap* gene cluster was subject to a phase-variation control mechanism (Low *et al.*, 1987). However, the question of the level at which pili regulation occurred (transcriptional versus post-transcriptional) was not addressed. To approach this problem, a fusion was made between *lac*ZYA and the pilin promoter. We chose plasmid pDAL17B-12A (constructed as shown in Figure 4 and described in Materials and methods) for fusion construction because it contained the complete pap_{17}I and pap_{17}B DNA sequence, but lacked pap_{17}A. The *pap*A gene appears to lack a promoter of its own (Figure 1 and Baga *et al.*, 1985) and has been shown to be co-transcribed with the *pap*B gene (Baga *et al.*, 1988). Therefore, pilin transcripts, which initiate at the *pap*BA promoter, should be measurable by assaying β-galactosidase production using this operon fusion. Plasmid pDAL17B-12A was digested with restriction endonuclease *Hin*dIII, end-filled and ligated to *Eco*RI linkers. Following restriction digestion with *Eco*RI, ligation with *Eco*RI-digested plasmid pRS551 was performed to construct the multicopy plasmid pDAL246B (Figure 4). Plasmid pDAL246B contains a pap_{17}B−*lac*ZYA operon fusion with the pap_{17}B promoter in the same orientation as *lac*Z. This plasmid was transformed into *E.coli* strain MC4100 and a transformant was transfected with the lambda phage λRS45 which carries homology to pRS551 (Simons *et al.*, 1987). A kanamycin resistant lysogen was selected (DL379). This lysogen resulted from recombination between λRS45 and pRS551 and was shown to contain a single integrated λ phage (λ246) using a Ter test (unpublished data, Mousset and Thomas, 1969). A pap_{21}′−*lac* operon fusion was made in a similar manner by using a deletion clone (pDAL261B) which contained pap_{21}I, pap_{21}B and a small portion of the pap_{21}A gene to construct fusion lysogen DL416 (see Materials and methods).

Escherichia coli strain DL379 was plated on minimal medium containing glycerol as a carbon source and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as an indicator of β-galactosidase activity (M9-Glycerol). Two distinct colony phenotypes were observed; colonies were either white (Lac$^-$) or blue (Lac$^+$). When DL379 Lac$^+$ colonies were replated on M9-Glycerol, ~35% of the progeny gave rise to Lac$^+$ colonies; whereas, DL379 Lac$^-$ colonies gave rise to ~0.5% Lac$^+$ colonies [Figure 5C and D; Table II; assuming an average colony size of 3.35×10^7 cells (25 generations)]. This indicated that pilin transcription was controlled by a heritable switch. In contrast, when strain MC4100 containing pDAL246B was examined on M9-Glycerol, all of the colonies were constitutively Lac$^+$ and no phase-variation was observed (Figure 5A). These results are consistent with our previous results which indicated that pilin phase-variation did not occur when pap_{17}I, pap_{17}B and pap_{17}A were present in multiple copies (Low *et al.*, 1987).

Table I. Bacterial strains, plasmids and phages used in this study

Strain, plasmid or phage	Description[a]	Reference and source
E.coli		
MC4100	F⁻ *ara*D139 Δ(*lac*IPOZYA-*arg*F)U169 *rps*L *thi*-1	Casadaban (1976)
DL379	MC4100 λ246 lysogen	this study
DL416	MC4100 λ261 lysogen	this study
DL430	MC4100 Nal^R	this study
ORN103	*thr*-1 *leu*-6 *thi*-1 Δ(*arg*F-*lac*)U169 *xyl*-7 *ara*-13 *mtl*-2 *gal*-6 *rps*L *fhu*A2 *min*A *min*B *rec*A13 Δ(*pil*ABCDFE *hyp*)	Orndorff *et al.* (1985)
DH5α	F⁻ *end*A1 *hsd*R17 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 Δ(*arg*F-*lac*)U169 φ80d*lac*ZΔM15	Bethesda Research Laboratories
Bacteriophages		
λRS45	*amp'*-*lac*ZYA imm^{21}	Simons *et al.* (1987)
λRS45-551	λRS45−pRS551 recombinant phage	this study
λ246	λRS45−pDAL246B recombinant phage	this study
λ261	λRS45−pDAL261B recombinant phage	this study
Plasmids		
pRS551	*amp-kan-lac*ZYA pMB1 replicon	Simons *et al.* (1987)
pTZ19U	*amp* pMB1 replicon	Mead *et al.* (1986)
pUC8	*amp* pMB1 replicon	Vieira and Messing (1982)
pDAL238B	*tet cam* P15A replicon with 2.7 kb pap_{21}IBA DNA sequence	Low *et al.* (1987)
pDAL246B	pRS551 containing a 1.6 kb pap_{17}IB DNA sequence	this study (Figure 2)
pDAL257B	pUC8 containing a 3.2 kb pap_{21}IBAH' DNA sequence	this study (Figure 2)
pDAL258B	pUC8 containing a 2.8 kb pap_{17}IBAH' DNA sequence	this study (Figure 2)
pDAL261B	pRS551 containing a 2.6 kb pap_{21}IBA' DNA sequence	this study (Figure 2)
pDAL17H-10	pUC8 containing a 2.0 kb pap_{17}IBAH' DNA sequence	this study (Figure 2)
pDAL17B-5	pUC8 containing a 1.2 kb pap_{17}I DNA sequence	this study (Figure 2)
pDAL17B-12A	pUC8 containing a 1.6 kb pap_{L17}IB DNA sequence	this study (Figure 4)
pDAL17B-23	pUC8 containing a 1.7 kb pap_{17}IB DNA sequence	this study (Figure 2)

[a]Resistance determinants: *amp*, ampicillin; *kan*, kanamycin; *tet*, tetracycline; *cam*, chloramphenicol.

DL416, containing the pap_{21}BA'−*lac*ZYA operon fusion in single copy, also gave rise to Lac^+ and Lac^- colonies after inoculation of M9-Glycerol medium. However, DL416 differed from DL379 because the Lac^- phenotype was 'leaky' (colonies were light blue) when cells were plated on M9-Glycerol. Furthermore, DL416 Lac^+ colonies gave rise to a much higher fraction of Lac^+ progeny when plated on M9-Glycerol (90%) compared to DL379 (35%, Table II). Interestingly, DL416 Lac^- colonies yielded a similar fraction (0.7%) of Lac^+ progeny compared to DL379 (Table II).

Calculation of phase transition rates using E.coli strains DL379 and DL416

The results shown above suggested that transcription initiated at the *pap*BA promoter was under the control of a heritable phase-variation mechanism. To further explore the nature of this phenomenon, we measured the rates at which $Lac^+ \longleftrightarrow Lac^-$ phase-variation occurred. In quantitating rates, a colony arising from either a Lac^+ or Lac^- parent cell was selected and the Lac phenotype of progeny cells was scored. The phase-transition frequency was calculated using the formula $(M/N)/g$ where M is the number of cells that underwent a phase-transition, N the total number of cells evaluated and g the total number of generations that gave rise to the colony (Eisenstein, 1981; see Materials and methods).

Using the method described above, we determined that the frequency of transition from $Lac^- \rightarrow Lac^+$ for DL379

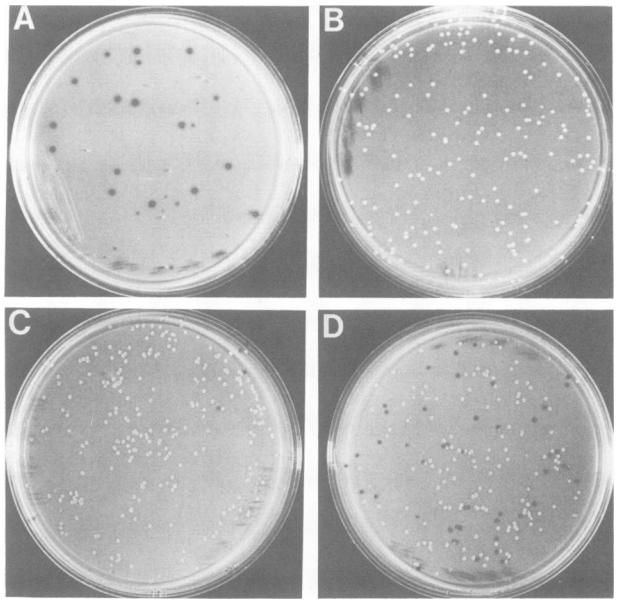

Fig. 5. Analysis of Lac phenotypes of *E.coli* K-12 strains carrying single copy pap_{17}'−*lac*ZYA fusions. A single colony of each of the strains listed below was inoculated onto M9-Glycerol media as described in Materials and methods. Plates were incubated for 48 h at 37°C prior to being photographed. Lac^+ and Lac^- colonies were derived from parent colonies showing no sectoring. Panels: **A**, MC4100 (pDAL246B); **B**, MC4100/λRS45-551 lysogen (transferred vector control); **C**, DL379 Lac^-; **D**, DL379 Lac^+.

Table II. Phase transition frequencies observed for *E.coli pap'-lac*ZYA fusion lysogens

*E.coli lac*ZYA φ lysogen	Lac phenotype/ carbon source[a]	Observed Lac$^+$ ⟷ Lac$^-$ switch frequencies			
		Total no. of colonies counted	No. of Lac$^+$/ no. of Lac$^-$ colonies	Switch frequency (α or β)[b]	Weighted average of frequencies[c]
DL379	Lac$^+$, M9-Glycerol	2398	953/1445	$\alpha = 2.45 \times 10^{-2}$	
	2nd colony as above	2123	805/1318	$\alpha = 2.51 \times 10^{-2}$	
	3rd colony as above	1951	520/1431	$\alpha = 2.89 \times 10^{-2}$	$\alpha = 2.60 \times 10^{-2}$
DL379	Lac$^-$, M9-Glycerol	3098	17/3081	$\beta = 2.22 \times 10^{-4}$	
	2nd colony as above	2517	4/2513	$\beta = 6.17 \times 10^{-5}$	
	3rd colony as above	2457	11/2446	$\beta = 1.74 \times 10^{-4}$	$\beta = 1.57 \times 10^{-4}$
DL416	Lac$^+$, M9-Glycerol	7014	6378/636	$\alpha = 3.72 \times 10^{-3}$	NA[d]
DL416	Lac$^-$, M9-Glycerol	9676	72/9604	$\beta = 3.05 \times 10^{-4}$	NA

[a]Carbon source refers to that included in solid media used for inoculation of parent and progeny colonies.
[b]The Lac$^+$ to Lac$^-$ switch frequency is designated α, and the Lac$^-$ to Lac$^+$ switch frequency is designated as β.
[c]The weighted average of the switch frequency was calculated as in Materials and methods and takes into account the number of progeny examined for each experiment used to calculate the average.
[d]Not applicable.

using M9-Glycerol was 1.57×10^{-4}/cell/generation (Table II). The frequency of transition from Lac$^+ \rightarrow$ Lac$^-$ was calculated to be 2.60×10^{-2}/cell/generation (Table II), which is 165-fold higher than the Lac$^- \rightarrow$ Lac$^+$ rate. Thus, after 25 generations a single Lac$^+$ bacterium will give rise to a colony (3.35×10^7 cells) which is comprised of mostly Lac$^-$ bacteria (65%); however, these colonies still display a Lac$^+$ phenotype (Figure 5D). In contrast, after 25 generations a single Lac$^-$ bacterium gives rise to a colony that is comprised of only 0.4% Lac$^+$ cells (Figure 5C). Measurement of phase transition rates for *E.coli* strain DL416 showed that the Lac$^+ \rightarrow$ Lac$^-$ rate was about 15-fold lower than the rate obtained using DL379. However, the Lac$^- \rightarrow$ Lac$^+$ rate was about double the rate obtained with DL379 (Table II).

Effect of carbon source and temperature on phase transition rates using E.coli strain DL379

Previous results indicated that transcription of the *pap* pilin gene of *E.coli* strain J96 was under catabolite repression control (Baga *et al.*, 1985). To determine if the carbon source might affect *pap* pili phase-variation, we measured phase transition rates for DL379 inoculated onto minimal medium containing glucose and X-gal (M9-Glucose). We found that the Lac$^+ \rightarrow$ Lac$^-$ transition rate was 3.85×10^{-2}/cell/generation whereas the Lac$^- \rightarrow$ Lac$^+$ transition rate was found to be 4.51×10^{-6}/cell/generation (Table III). This represents a 37 000-fold difference between transiton rates. As only three Lac$^+$ colonies were observed from a total of 119 000 progeny cells examined, the Lac$^- \rightarrow$ Lac$^+$ transition rate was only an approximation. To ensure that the Lac$^-$ colonies obtained on M9-Glucose were capable of phase-variation, random colonies were picked to M9-Glycerol. These colonies gave rise to both Lac$^+$ and Lac$^-$ progeny (our unpublished data). Comparing the data obtained using M9-Glucose to that obtained using M9-Glycerol, the Lac$^+ \rightarrow$ Lac$^-$ transition rate using glucose was 1.4-fold higher than results obtained using glycerol. The Lac$^- \rightarrow$ Lac$^+$ rate using M9-Glucose was about 35-fold lower than the rate obtained with M9-Glycerol (Table III). Taken together, these data showed that the carbon source had a profound effect on the Lac$^- \rightarrow$ Lac$^+$ phase transition rate but only a small effect on the Lac$^+ \rightarrow$ Lac$^-$ rate.

Pap pilin transcription has been shown to be reduced by about 25-fold at an incubation temperature of 23°C compared to an incubation temperature of 37°C (Goransson and Uhlin, 1984). This study was performed using multicopy *pap* DNA sequences which, as discussed above, do not allow an analysis of *pap* pilin phase-variation. To determine the effects of incubation temperature on *pap* pilin phase switching, we inoculated strain DL379 cells, initially incubated at 37°C, onto M9-Glycerol at 23, 30 and 37°C. Lac$^+$ ⟷ Lac$^-$ phase transition rates were similar at 30 and 37°C (Table IV). To ensure that the temperature shift itself was not affecting phase transition rates, we also incubated DL379 at 30°C and incubated progeny cells at 30°C. Similar results were obtained compared with the 37→30°C temperature shift experiment (Table IV). Interestingly, when DL379 cells (Lac$^+$ or Lac$^-$) were incubated at 37°C and then progeny were incubated at 23°C, all colonies displayed a Lac$^-$ phenotype. Previous work has suggested that β-galactosidase is at least as active at 23°C as it is at 37°C (Goransson and Uhlin, 1984). In addition, we have isolated *E.coli* (pap_{17}' *-lac*) mutants that display a phase-variation phenotype at 23°C, demonstrating that the Lac$^+$ phenotype can be expressed at low temperature (C.White-Ziegler and D.Low, in preparation). Therefore, our results suggest that the differences in phase transition rates observed at 23°C are due to an alteration in transcription rather than β-galactosidase activity.

Discussion

The results presented here strongly indicate that *pap* pili phase-variation occurs at the transcriptional level. However, we have not ruled out the possibility that regulation of phase-variation might occur post-transcriptionally due to differential rates of mRNA degradation or differences in translational efficiency. Our results also indicate that the phase of cells (Lac$^+$ or Lac$^-$ in our study) is a heritable trait since

Table III. The effect of carbon source on observed phase-transition frequencies for *E.coli* strain DL379

Lac phenotype/ carbon source[a]	Observed $Lac^+ \longleftrightarrow Lac^-$ frequencies			
	No. of colonies counted	Lac^+/Lac^- ratio[b]	Transition frequency (α or β)[c]	Weighted average of frequencies[d]
Lac^+, M9-Glycerol	Table II	Table II	Table II	$\alpha = 2.60 \times 10^{-2}$
Lac^-, M9-Glycerol	Table II	Table II	Table II	$\beta = 1.57 \times 10^{-4}$
Lac^+, M9-Glucose	2078	61/2017	$\alpha = 3.82 \times 10^{-2}$	
2nd colony as above	2327	239/2088	$\alpha = 3.73 \times 10^{-2}$	
3rd colony as above	2176	122/2054	$\alpha = 3.94 \times 10^{-2}$	
4th colony as above	2485	142/2343	$\alpha = 3.90 \times 10^{-2}$	$\alpha = 3.85 \times 10^{-2}$
Lac^-, M9 Glucose	27086	0/27086	NA[e]	
2nd colony as above	64172	0/64172	NA	
3rd colony as above	27656	3/27653	$\beta = 4.51 \times 10^{-6}$	NA

[a]Solid media containing glucose or glycerol as sole carbon source were inoculated with *E.coli* strain DL379.
[b]Lac^+/Lac^- ratio is the number of Lac^+ colonies divided by the number of Lac^- colonies.
[c]The $Lac^+ \rightarrow Lac^-$ transition frequency is designated α, and the $Lac^- \rightarrow Lac^+$ transition frequency is designated β.
[d]The weighted average of the switch frequency was calculated as in Materials and methods and takes into account the number of progeny examined for each experiment.
[e]Not applicable.

Table IV. The effect of temperature on observed transition frequencies for strain DL379

Lac phenotype of colony analyzed	Incubation temperature (°C)		Observed $Lac^+ \longleftrightarrow Lac^-$ switch frequencies		
	Primary[a]	Secondary	Total number of colonies counted	No. of Lac^+/Lac^- colonies	Switch frequency (α or β)[b]
Lac^+	37	37	Table II	Table II	$\alpha = 2.60 \times 10^{-2}$[c]
Lac^-	37	37	Table II	Table II	$\beta = 1.57 \times 10^{-4}$
Lac^+	37	30	1571	549/1022	$\alpha = 2.65 \times 10^{-2}$
Lac^-	37	30	3634	17/3617	$\beta = 1.88 \times 10^{-4}$
Lac^+	37	23	1459	0/1459	NA[d]
Lac^-	37	23	1957	0/1957	NA
Lac^+	30	30	5196	1736/3460	$\alpha = 2.77 \times 10^{-2}$
Lac^-	30	30	8287	56/8231	$\beta = 2.71 \times 10^{-4}$

[a]Primary refers to the initial plate from which the colony was picked; secondary refers to the plates that the colony was transferred to for quantitation of switch frequencies. All plates were M9-Glycerol.
[b]The Lac^+ to Lac^- switch frequency is designated α, and the Lac^- to Lac^+ switch frequency is designated as β.
[c]These switch frequencies from Table II are presented as the weighted average.
[d]Not applicable. Because there were no phase-variation events at 23°C we were unable to calculate a switch frequency.

bacteria from a Lac^+ colony gave rise to significantly more Lac^+ colonies than bacteria from Lac^- colonies after reinoculation of M9-Glycerol medium (Figure 5 and Table II). Phase-variation was not observed for *E.coli* containing the multicopy *pap'−lac* operon fusion plasmids pDAL246B (Figure 5A) or pDAL261B (our unpublished data). Transfer of *pap'−lac* DNA sequences to a λ prophage resulted in the appearance of the phase-switching phenotype (Figure 5C and D; Table II). These results are consistent with our previous work showing that virutally all *E.coli* K-12 cells harboring multicopy plasmids containing the *pap*-17 operon expressed pilin-17. In contrast, *E.coli* harboring a single copy *pap*-17 operon displayed *pap* pili phase-variation (1% of cells were pilin-17^+) similar to that observed for the uropathogenic *E.coli* strain C1212 (6% of cells were pilin-17^+, Low *et al.*, 1987). Thus, normal (wild type) *pap* pili phase-variation is abrogated in cells containing multicopy *pap* DNA sequences.

Pap pili phase-variation appears to occur at both the transcriptional level (this study) and pili expression level (Low *et al.*, 1987). It has been recently shown that the *pap*BA transcript is subject to post-transcriptional processing (Baga *et al.*, 1988). Therefore, it is possible that the phase transition rates measured here (Tables II and III) might differ from pili expression phase transition rates. Previously, we did not measure phase transition rates for *pap* pili expression due to the difficulties in analyzing individual colonies. Instead, we measured the fraction of cells within an *E.coli* strain C1212 colony that expressed pilin-17 (6%) and pilin-21 (84%). It is difficult to compare this pili expression data with the phase transition frequencies obtained here because the former data were obtained without knowledge of the *pap* pili phase ('phase on' or 'phase off') of the starting colonies. Also, the pili expression data were obtained using LB agar (Low *et al.*, 1987) whereas the phase transition frequencies shown here (Table II) were obtained using M9-Glycerol minimal medium (this study).

The pap_{17}IB and pap_{21}IB DNA regulatory regions (Figure 1) are very similar to each other and to the *pap*IB regulatory sequence of *E.coli* strain J96 (Baga *et al.*, 1985).

Interestingly, the $Lac^+ \rightarrow Lac^-$ transition frequency for *pap*-17 (DL379) is about 15-fold higher than the frequency measured for *pap*-21 (DL416), whereas the $Lac^- \rightarrow Lac^+$ transition frequencies differed by less than 2-fold (Table II). What DNA sequence changes between *pap*-17 and *pap*-21 are the cause of this difference in transition frequencies? The PapB protein sequences were identical and there was only one amino acid difference for PapI (Figure 1). However, there were 23 bp differences in the intergenic region between *papI* and *papB*, including a 9 bp insertion present in *pap*-21 but not *pap*-17. We do not known the role(s), if any, of these base-pair and amino acid changes in the transition frequency differences observed between the *pap*-17 and *pap*-21 operons. In addition, the *pap'−lac* fusions used in this study also contained ~300 bp (*pap*-17) and 600 bp (*pap*-21) of DNA downstream of *papI* that we have not sequenced and could play a regulatory role in *pap* pili expression. Although it is possible that these unsequenced DNA regions encode protein(s), none were detected using *E.coli* minicell analysis (Figure 3).

Our results in Figure 3 showed that both the *pap*-17 and *pap*-21 DNA sequences used to construct *pap'−lac* operon fusions encoded proteins (PapI and PapB) of mol. wts similar to those predicted from the DNA sequence (Figure 1). Previous results have indicated that PapB, and to a lesser extent PapI, are positive activators, increasing transcription from the *pap*BA promoter (Baga *et al.*, 1985). However, we do not know what role(s), if any, are played by these proteins in the phase-variation process. Previously described phase-variations systems, including *Salmonella* flagellar phase-variation (Silverman and Simon, 1980; Zieg and Simon, 1980) and related systems (Plasterk and van de Putte, 1984; Plasterk *et al.*, 1983), in addition to type 1 pili phase-variation (Abraham *et al.*, 1985) utilize a DNA inversion event to control transcription. We do not know if *pap* phase-variation also involves a DNA rearrangement, although we have not been able to detect any major DNA rearrangements between Lac^+ and Lac^- *E.coli* strain DL379 cells (unpublished data).

Previous results showed that transcription initiated at the *pap*BA promoter was subject to catabolite repression via cAMP−CRP interaction (Baga *et al.*, 1985). Our results showed that the $Lac^- \rightarrow Lac^+$ transition frequency of *E.coli* strain DL379 inoculated onto M9-Glucose was reduced by ~35-fold compared to cells inoculated onto M9-Glycerol medium (Table III). However, the $Lac^+ \rightarrow Lac^-$ transition rate for strain DL379 inoculated onto M9-Glucose medium was 1.4-fold higher than for cells inoculated onto M9-Glycerol (Table III). Our interpretation of these results is that the carbon source used for growth, possibly via cAMP−CRP control, influences the rate of transition from a 'phase-off' state to a 'phase-on' state. Once a bacterial cell is in the 'phase-on' state, carbon source has only a small effect on the rate of transition to the 'phase-off' state. The end result of this phenomenon is to shift the bacterial population towards a 'phase-on' or 'phase-off' state through a heritable mechanism, although it is not yet clear what role(s) this response might have in the normal physiology of *E.coli* living in the bowel or outside of a living host.

In contrast to the influence of carbon source on *pap* pili phase switching, low temperature (23°C) appears to induce a complete transition from the 'phase-on' state to the 'phase-off' state (Table IV). Previous results indicated that transcription from the *pap*BA promoter of strain J96 was reduced ~25-fold at 23°C compared to 37°C (Goransson and Uhlin, 1984). These results were obtained using *E.coli* strains containing multicopy *pap* DNA sequences which, as we have shown here, do not display the normal phase-variation phenotype (Figure 5A). Thus, although the results show a similar general effect of temperature on *pap* pilin transcription, different parameters are being measured by the multicopy and single copy *pap* systems. Whereas the single copy *pap'−lac* system allows measurement of phase-variation, the multicopy system provides a measurement of transcription which is an average for the whole bacterial population being measured. As shown here, this measurement does not accurately reflect the state of wild-type cells in which *pap* is in a single copy state.

It seems that at least one function of the low temperature response is to turn off the production of pili by *E.coli* outside of a living host where presumably the pili are not needed and may actually be detrimental. By similar reasoning, other factors that might be needed for colonization of the intestine may also be regulated by a low temperature response system and thus could be identified by a low temperature response phenotype.

Materials and methods

Bacterial and bacteriophage strains, plasmids and media

Strains, plasmids and bacteriophages are listed in Table I. LB broth, LB agar, M9 minimal broth and M9 minimal agar were prepared according to Miller (1972). When used, supplements were at the following concentrations: glucose and glycerol, 0.2% final concentration; 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 40 μg/ml final concentration; ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; and nalidixic acid, 20 μg/ml.

Calculation of phase transition rates

$Lac^+ \rightarrow Lac^-$ and $Lac^- \rightarrow Lac^+$ transition rates were calculated by a modification of the method of Eisenstein (1981). Fusion strains were inoculated onto M9 agar containing the appropriate carbon source. Colonies showing a complete Lac^+ or Lac^- phenotype (no sectors) were excised from the agar and resuspended at 4°C in M9 salts containing no carbon source. The total number of organisms was determined with the aid of a hemocytometer and appropriate dilutions were inoculated onto M9 agar containing the same carbon source as the parent colony. After ~48 h of growth, the colonies were scored for Lac phenotype. Colonies showing greater than 50% Lac^+ phenotype were scored as Lac^+, and all others were scored as Lac^-.

Transition rates were calculated by the formula $(M/N)/g$ where 'M/N' is the ratio of Lac^+ cells to total cells or the ratio of Lac^- cells to total cells, and 'g' is the number of generations of growth from a single cell to the total number of cells in the colony. The weighted average of the transition rates was calculated by the formula $[(M_1/g_1) + (M_2/g_2) + (M_n/g_n)]/(N_1 + N_2 + \ldots N_n)$ where M, N and g are as above and n represents each individual transition rate calculation. In order to calculate the transition rates the assumption was made that Lac^+ colonies arose from a single Lac^+ parent cell and Lac^- colonies arose from a single Lac^- parent cell.

Recombinant DNA techniques and identification of plasmid-encoded polypeptides

Conditions for restriction endonuclease mapping, isolation of restriction fragments, ligation and transformation of plasmid DNA have been described previously (Low *et al.*, 1987; Maniatis *et al.*, 1982).

Plasmid deletion subclones pDAL17H-10, pDAL17B-5, pDAL17B-12A and pDAL17B-23 were constructed using DNase I as follows. Plasmid pDAL258B DNA (2 μg) was digested with DNase I (Worthington Biochemicals) at 22°C to yield ~0.5 double stranded cleavages per plasmid circle. DNase I digestion was performed in a buffer containing 20 mM Tris−HCl pH 7.6 and 10 mM $MnSO_4$. After addition of EDTA to a final concentration of 25 mM to prevent further DNase I digestion, DNA samples were analyzed on Tris−acetate agarose gels (Maniatis *et al.*, 1982). Full-

sized linear DNA fragments were cut out of the gel and concentrated using a Gene Clean kit (Bio101, Inc.). DNA samples were digested with *Bam*HI or *Hin*dIII, which each have a single recognition site within the pUC8 vector DNA sequence. DNA fragments were end-filled using the Klenow fragment of DNA polymerase I, concentrated with the Gene Clean kit, and incubated with T4 DNA ligase under dilute conditions to favor intramolecular joining. Ligation mixtures were added to transformation-competent *E.coli* strain DH5α cells and inoculated onto LB agar medium containing ampicillin (100 μg/ml). Transformants were picked at random and contained plasmids with deletions extending into the *pap* sequence from either the *Bam*HI or EcoRI restriction endonuclease recognition site of vector pUC8.

Plasmid-encoded polypeptides were identified as previously described (Normark *et al.*, 1983; Low *et al.*, 1987) using the *E.coli* minicell strain ORN103 (Orndorff *et al.*, 1985). The ^{35}S-labeled *pap*-encoded polypeptides were separated on a SDS−PAGE system designed for the separation of low mol. wt polypeptides (Giulian *et al.*, 1985). Gels were treated with En^{3}Hance (New England Nuclear) to increase signal intensity and were soaked in a solution containing 10% polyethylene glycol (PEG 6000) in water prior to drying to prevent expansion.

Construction of pap′−lacZYA operon fusions

A pap_{17} operon fusion was constructed as shown in Figure 4 using plasmid vector pRS551 (Simons *et al.*, 1987). The resulting pDAL246B plasmid contained a pap_{17}′−*lac*ZYA fusion with the *lac* genes under the control of the *pap*BA promoter.

A pap_{21} operon fusion was constructed as follows. The 2.7 kb *Bam*HI−*Cla*I DNA fragment from pDAL238B (Low *et al.*, 1987) was ligated to *Bam*HI−*Cla*I digested phage M13 mp18 DNA (Yanisch-Perron *et al.*, 1985) to construct phage M13-243B. Phage M13-243B was used to generate deletions extending in from the pilin gene region using CYCLONE 1 as described above. One clone (M13-L1P52) was chosen which contained pap_{21}I, pap_{21}B and ~185 bp of the pap_{21}A (pilin) DNA sequence. Phage M13-L1P52 was digested with *Hin*dIII and end-filled using the Klenow fragment of DNA polymerase I and deoxynucleotide triphosphates (Ausubel *et al.*, 1987). Following a 10 min incubation at 70°C to inactivate the Klenow fragment of DNA Pol I, *Eco*RI linkers d(pCGGAATTCCG) (New England BioLabs) and T4 DNA ligase were added. Following incubation for 16 h at 15°C, *Eco*RI was added for 2 h at 37°C and then loaded onto a low melting temperature (LMT) agarose gel (0.7%, FMC BioProducts). The 2.4 kb DNA fragment containing *pap* DNA sequences was excised and ligated to *Eco*RI-digested, calf intestinal phosphatase-treated plasmid pRS551. This ligation mixture was used to transform competent *E.coli* strain DH5α cells with selection on LB agar plates containing ampicillin (100 μg/ml) and X-Gal (Silhavy *et al.*, 1984). Dark blue colonies harbored vector pRS551 containing the 2.4 kb *pap* DNA fragment in the orientation which placed *lac*ZYA under control of the *pap*BA promoter. This construct was designated as pDAL261B.

Pap′−lac fusions were transferred from plasmids pDAL246B and pDAL261B to phage λ as described previously (Simons *et al.*, 1987). In addition, a 'transferred vector' control was prepared using plasmid pRS551. Briefly, plasmids were first transformed into strain MC4100 (Casadaban, 1976) and used as host for the growth of phage λRS45 (Simons *et al.*, 1987). Phage lysates were used to infect a nalidixic acid derivative of strain MC4100 (DL430) and bacterial cultures were inoculated onto LB agar medium containing kanamycin (25 μg/ml) and nalidixic acid (20 μg/ml). Colonies arising were cross-streaked with λ*imm*^{21} phage to verify the lysogenic state of the bacteria. Prophage copy number was determined using a Ter test (Mousset and Thomas, 1969).

DNA sequence analysis

DNA sequence analysis was performed using the dideoxy termination method of Sanger *et al.* (1977). Deletion subclones were generated using the CYCLONE I biosystem kit (International Biotechnologies, Inc.) or by subcloning DNA restriction fragments into plasmid pTZ19U (Mead *et al.*, 1986). Alternatively, deletion subclones were generated by a procedure using DNase I as follows. DNA restriction fragments were cloned into the vector pUC8 and incubated in the presence of manganese with an amount of DNase I sufficient to generate about one double stranded cleavage per DNA molecule. After separation on agarose gels, linear DNA fragments were digested with *Bam*HI, which cleaves once within the plasmid vector. After end-filling with the Klenow fragment of DNA polymerase I, linear DNA fragments were circularized by addition of T4 DNA ligase at 15°C for 16 h. Resulting ligation products were transformed into *E.coli* strain DH5α (Bethesda Research Laboratories) and transformants appearing on LB agar medium containing ampicillin (100 μg/ml) were picked. Plasmids were examined by agarose gel electrophoresis and appropriate deletion derivatives were subjected to DNA sequence analysis.

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