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Environmental temperature regulates transcription of a virulence pili operon in *E. coli*

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The expression in *Escherichia coli* K-12 of a pilus-adhesion determinant, obtained through molecular cloning from a pyelonephritic *E. coli* isolate, was studied at different temperatures. Strain HB101 carrying the recombinant plasmid pRHU845 agglutinated human erythrocytes after growth at 37°C but not after growth at 22°C. Quantitation of pilus subunit protein by an enzyme-linked immunosorbent assay (ELISA) for pilus antigen showed that synthesis of the pilus subunits was reduced at least 20-fold at 22°C as compared with 37°C. The 5′ end of the pilus subunit structural gene, *papA*, was fused to the *lacZ* gene such that expression could be monitored at both translational and transcriptional levels. Measurements of β-galactosidase production by the *papA-lacZ* hybrids provided evidence for thermoregulation of *papA* gene transcription. A regulatory determinant was localized to a 2-kb *Eco*RI-*Hind*III fragment encoding the *papB* gene and part of *papA* although none of the presently known *pap* gene products seem to be directly involved in a thermoregulatory mechanism. Comparison with other thermoregulatory systems in *E. coli* suggests that *pap* gene expression is regulated by a novel mechanism.

Key words: bacterial pathogenicity/gene expression/hemagglutination/*lacZ* fusion/thermoregulation

Introduction

Pathogenic bacteria are characterized by their ability to express a number of special functions which non-pathogenic bacteria lack (Falkow, 1979). For example, *Escherichia coli*, which is the most common enteric bacterium causing extraintestinal infections in man, may express adherence factors (Beachey, 1981), hemolysins (Cooke and Ewins, 1975; Welch *et al.*, 1981), specific O and K antigens (Smith and Huggins, 1980; Silver *et al.*, 1981), and additional iron-sequestering systems (Williams, 1979). However, little is known about the molecular mechanisms determining expression of such virulence factors. Several virulence functions appear to be influenced by the growth temperature of the bacteria. Production of K1 capsular antigen (Bortolussi *et al.*, 1983) and adherence factors mediating colonization by enteropathogenic *E. coli* (Evans *et al.*, 1977; de Graaf *et al.*, 1980) or *Salmonella typhimurium* (Jones and Richardson, 1981), as well as expression of invasive virulence by pathogenic *Shigella* (Maurelli *et al.*, 1984) and *Yersiniae* (Brubaker, 1979) have been shown to depend on environmental temperature. The majority of *E. coli* strains isolated during acute pyelonephritis from children with unobstructed urinary tracts can agglutinate human erythrocytes in a mannose-resistant fashion (Hagberg *et al.*, 1981). Hemagglutination appears to depend on expression of fimbrial appendages (pili) that are also thought to mediate attachment to uroepithelial cells (Källenius *et al.*, 1980). By molecular genetic analysis of such a virulence function it should be possible to define the regulatory mechanism(s) involved.

The pyelonephritic *E. coli* isolate, J96, expresses mannose-resistant hemagglutination (MRHA) of human erythrocytes and production of Pap pili (pili associated with pyelonephritis) on the bacterial cell surface (Hull *et al.*, 1981). By recombinant DNA technology the genetic determinant for this virulence property has been isolated and characterized (Normark *et al.*, 1983; Norgren *et al.*, 1984). An 11-kbp DNA fragment carried by plasmid pRHU845 was shown to confer the binding properties and pili production on the *E. coli* K-12 laboratory strain HB101. Here we present evidence that transcription of the structural gene for Pap pili is regulated in response to environmental temperature.

Results

Effect of temperature on Pap antigen expression

While characterizing the Pap pili-adhesion gene cluster from strain J96 we found that pili antigen and ability to hemagglutinate (Table I) were absent when bacteria (HB101/pRHU845) were cultured at 20 – 22°C rather than 37°C. A quantitative analysis of Pap antigen produced by strain HB101 carrying the Pap plasmid pRHU845 was performed using a competitive ELISA (Normark *et al.*, 1983). The Pap pili are polymers of a 19 500 daltons pilin polypeptide encoded by the *papA* gene (Normark *et al.*, 1983).To distinguish between an effect of temperature at the level of pilin subunit synthesis as compared with pilus assembly, we determined the relative amounts of surface-exposed and total Pap antigen at different temperatures. As shown in Table II, growth at 30°C or 22°C reduced the amounts of surface-exposed and total Pap antigen in parallel, and at 22°C the ELISA response was not much different from that of the plasmid-free control strain at 37°C.

Regulation of β-galactosidase expression in a papA-lacZ gene fusion

That there was no appreciable accumulation of the major

Table I. Effect of growth temperature on hemagglutination-phenotype

Strain	MRHA after growth at[a]:	
	37°C	22°C
HB101	–	–
HB101/pRHU845	+	–
HB101/pSNO25	–	–
HB101/pHMG73	+	–
RW193/pRHU845	+	–
RW193/pACYC184	–	–
UT4400/pRHU845	+	–
UT4400/pACYC184	–	–

[a]Bacteria grown on solid media (LA-plates) were assayed for hemagglutination of human erythrocytes in the presence of α-methyl-D-mannoside as described before (Norgren *et al.*, 1984). MRHA = mannose-resistant hemagglutination.

Table II. Expression of Pap antigen at different growth temperatures[a]

Strain	Growth temperature (°C)	Relative amount of Pap antigen[b]: Surface exposed	Cell extracts
HB101/pRHU845	37	1	1
HB101/pRHU845	30	0.32	0.37
HB101/pRHU845	22	0.03	0.06
HB101	37	0.02	<0.01

[a]*E. coli* strains HB101/pRHU845 and HB101 (Pap$^-$ control) were grown on solid medium (LA-plates). After harvest the cells were subjected to competitive ELISA for quantitation of Pap antigen. Assays were carried out with intact cells to determine the amount of surface-exposed Pap antigen and with cell extracts to determine the total amount. Anti-Pap rabbit antiserum and procedures for making the extracts were as described before (Normark *et al.*, 1983).
[b]The amount of Pap antigen is expressed relative to the amount at 37°C for HB101/pRHU845 cells and extracts, respectively, and represents the mean of four assays using different concentrations of bacteria.

Pap antigen (i.e., the PapA protein) at 22°C suggested that the temperature effect operates at the level of pilin subunit synthesis. A fusion between the *papA* and *lacZ* genes was constructed by inserting the 2-kb *Eco*RI-*Hind*III fragment of plasmid pRHU845 (Norgren *et al.*, 1984; see Figure 1) into the fusion vector pSKS107 (Shapira *et al.*, 1983). The fusion plasmid pBEU80 (see Figure 1) therefore offered the possibility to test if β-galactosidase expression by the *papA-lacZ* hybrid gene was temperature dependent. A culture of strain MC1061/pBEU80 growing at 30°C was divided into three portions and the β-galactosidase synthesis was monitored at 20, 30 and 37°C. As shown in Figure 2, the rate of synthesis was drastically increased by the shift from 30 to 37°C whereas there was a decrease after the shift down to 20°C. The effect of growth temperature on the *papA-lacZ* expresion was then compared with the expression from *lacZ*$^+$ plasmids carrying the *lac* regulatory region (plasmid pSKS106) or the *tet* promoter and *trpA* translational initiation signal (pHMG2). Steady-state levels of β-galactosidase activity were determined in cultures at 37, 30 and 22°C (Table III). Whereas the expression from plasmid pBEU80 was decreased to ~40% at 30°C and 15% at 22°C as compared with the 37°C level there was an increased expression at the lower temperatures from the other two plasmids. Although the extent of the observed reduction in *papA-lacZ* expression might also have been influenced by growth rate and temperature effects on plasmid copy number (Engberg and Nordström, 1975), it was evident that the *papA-lacZ* hybrid gene behaved very differently from the other genes.

Thermoregulation of transcription

The 2-kb *Eco*RI-*Hind*III fragment encoding the 5′ end of *papA* was also utilized for analysis of the effect of growth temperature on *papA* transcription. A transcriptional *papA-lacZ* fusion (see plasmid pHMG1 in Figure 1) was constructed in the promoter-probing vector pRZ5202. The vector carries the β-lactamase gene, which mediates ampicillin resistance, and we used this as an internal control to monitor changes in plasmid gene dosage, and thereby plasmid copy number (Uhlin and Nordström, 1977). As shown in Table IV, the ratio of β-galactosidase to β-lactamase produced by strain MC1061/pHMG1 was more than 10-fold lower at 23°C than at 37°C. However, the vector itself (pRZ5202) expressed β-galactosidase activity at both temperatures. After correcting for the background activity expressed by the vector we calculated that *papA* expression by pHMG1 at 23°C was ~4% of

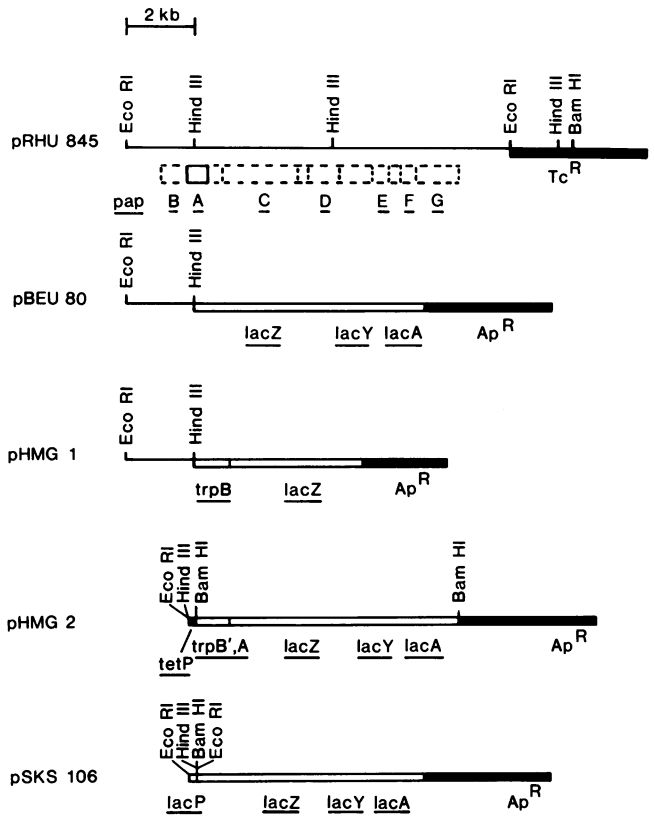

Fig. 1. Physical and genetic organization of the Pap plasmid pRHU845 and Lac fusion plasmids. Above the linear representations of the different plasmids the positions of the recognition sites for restriction endonucleases *Bam*HI, *Eco*RI and *Hind*III are shown. The single lines in pRHU845, pBEU80 and pHMG1 represent Pap DNA. Positions of the *pap* cistrons (A–G) in pRHU845 are indicated by dashed line boxes. The *papA* gene (solid line box) encodes the Pap pili subunit protein and has recently been sequenced (Båga *et al.*, 1984). Analysis of transposon Tn*5* insertion mutants suggested that the *pap* genes were all transcribed in the direction from B to G on the map (Norgren *et al.*, 1984). The vector replicon was pACYC184 (Chang and Cohen, 1978) in pRHU845 and pBR322 (Bolivar *et al.*, 1977) in the other plasmids. Ap^R and Tc^R denote resistance to ampicillin and tetracycline, respectively. The Lac fusion plasmids, pSKS107 (Shapira *et al.*, 1983) and pRZ5202 (kindly provided by W.S.Reznikoff), were used in construction of translational (pBEU80) and transcriptional (pHMG1) *papA-lacZ* fusions. Plasmid pHMG2 was constructed by inserting a *Bam*HI fragment (carrying the *lac* genes) from pMC903 (Casadaban *et al.*, 1980) into the *Bam*HI site of pBR322 such that *lacZ* transcription is controlled by the tetracycline-resistance gene promoter. pSKS106 carries the *lac* promoter and is otherwise similar to pSKS107 (Shapira *et al.*, 1983).

that at 37°C. This value is in good agreement with the result from quantitation of Pap antigen by the ELISA (Table II). We therefore conclude that the temperature effect is exerted at the level of *papA* transcription.

We know from analysis of Tn*5* insertion mutants of pRHU845 that a number of cistrons (*papA–F*, see Figure 1) are involved in the biogenesis of Pap pili and expression of MRHA (Norgren *et al.*, 1984). The *papB* gene encodes a 13 000-dalton polypeptide and is located adjacent to *papA* within the 2-kb *Eco*RI-*Hind*III fragment used for *lacZ* fusions. A mutant (pSNO25) of plasmid pRHU845 carrying the transposon Tn*5* in the *papB* gene showed reduced expression of Pap antigen and a negative MRHA (Norgren *et al.*, 1984). In order to assess the role of *papB* we have begun experiments to create non-polar mutations in the *papB* gene. As a preliminary analysis of whether or not *papB* is involved in the temperature regulation we tested one such mutant (pHMG73)

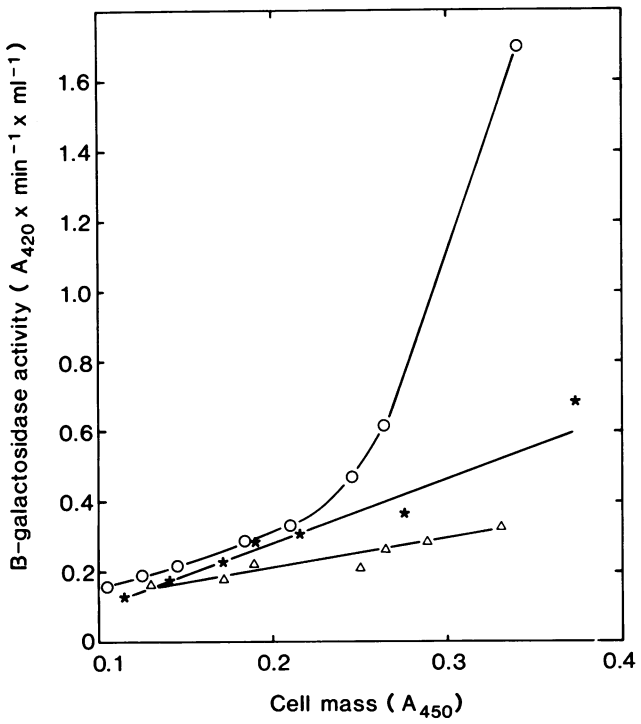

Fig. 2. Differential rate of synthesis of β-galactosidase by the *papA-lacZ* hybrid pBEU80. A logarithmically growing broth-culture of strain MC1061/pBEU80 at 30°C was diluted and divided into three portions which were incubated at 37°C (circles), 30°C (crosses) or 20°C (triangles). Starting after one doubling in cell mass at the post—shift temperature, samples were withdrawn at intervals for assay of β-galactosidase activity. Cell growth was monitored by optical density measurements (at 450 nm) and the growth rate (doublings/h) at 37, 30 and 20°C were 1.7, 1.0 and 0.4, respectively. The bacteria were grown in the absence of antibiotic selection. At the end of the experiment, samples were spread on LA-plates to test for plasmid segregation (see Materials and methods). The frequencies of plasmid-carrying cells were 100, 96 and 92% at 37, 30 and 20°C, respectively.

Table III. Effect of growth temperature on β-galactosidase production by *lacZ* fusion plasmids

Plasmid in strain MC1061	*lacZ* under control of	Relative enzyme activity at[a]: 37°C	30°C	22°C
pBEU80	*pap*	1	0.42	0.14
pSKS106	*lac*	1	1.66	1.83
pHMG2	*tet*	1	1.48	1.57

[a]Bacteria were grown in broth medium at different temperatures and assayed for specific activity of β-galactosidase (Miller, 1972). The activity is expressed relative to the value at 37°C which was 232, 7506 and 566 units/A_{420} = 1 for pBEU80, pHMG2 and pSKS106, respectively.

for expression of MRHA at 37°C and 22°C (Table I). The result indicated that the *papB* gene product is not involved, but the comparison between pSNO25 and pHMG73 at 37°C suggested that the transposon in *papB* might cause a polarity effect on other *pap* genes in the case of pSNO25.

The relative amounts of the OmpF and OmpC porins in *E. coli* vary with growth temperature such that decreased temperature results in increased amounts of OmpF protein and correspondingly decreased quantities of OmpC protein (Lugtenberg *et al.*, 1976; Lundrigan and Earhart, 1984). Recently, the gene *envY* was suggested to encode a regulatory protein controlling this temperature-dependent fluctuation in porin proteins (Lundrigan and Earhart, 1984). To test if the EnvY

Table IV. Analysis of a transcriptional *papA-lacZ* fusion[a]

Strain	Growth temperature (°C)	β-galactosidase activity (units)	β-lactamase activity (arbitrary units)	Ratio β-gal/ β-lact
MC1061/pRZ5202	37	12.7	1	12.7
MC1061/pRZ5202	23	16.0	0.99	16.2
MC1061/pHMG1	37	241.0	0.85	283.5
MC1061/pHMG1	23	16.9	0.65	26.0

[a]Cultures in steady-state at 37°C and 23°C were assayed for activity of the enzymes β-galactosidase and β-lactamase (see Materials and methods).

protein would be involved in the thermoregulation of *pap* gene expression we introduced the Pap$^+$ plasmid pRHU845 (and the vector pACYC184 as control) into an *envY* mutant strain which was defective in thermoregulation of the porins. The *envY* mutant host (UT4400) and the otherwise isogenic *envY*$^+$ strain (RW193) were similar with respect to temperature effect on hemagglutination (Table I). The thermoregulation of *pap* gene expression therefore appears to be different from the regulatory system involved in porin expression.

Discussion

The growth temperature has a pronounced effect on expression of Pap pili and on bacterial adhesion to human cells. By an immunoassay we could demonstrate that the amount of Pap pili subunits in *E. coli* is very much reduced after growth at temperatures <37°C (Table II). Experiments with *lacZ* fusions in the pili subunit structural gene, *papA*, showed that the thermoregulation operates at the level of gene transcription. It was also evident that a regulatory determinant must be contained within the 2-kb *Eco*RI-*Hind*III fragment which encodes the N-terminal part of the PapA protein. Results from preliminary studies of a mutant in which the *papB* gene was inactivated suggested that this putative regulatory gene might not be involved in the thermoregulation. It therefore appears as if none of the known *pap* gene products directly takes part in the regulatory system. The K88 and K99 fimbrial antigens of enterotoxigenic *E. coli* are also absent when bacteria are grown at 18–20°C (Gaastra and de Graaf, 1982). Analysis of K99 expression indicated that the subunits are not synthesized at 18°C and it was proposed that either initiation or chain elongation of subunit synthesis requires a membrane site which might be altered by a temperature change (Isaacson, 1983). Having established by molecular genetic methods that *papA* expression is regulated at the transcriptional level we find it more likely that temperature affects a regulatory sequence or other component(s) involved in the initiation of transcription.

By analyzing synthesis of *pap* gene products in the minicell system we have obtained indications that, in addition to the PapA protein, the *papB, papE, papF* and *papG* gene products are produced in lower amounts at 25°C as compared with 37°C (unpublished data). In the case of *papC* and *papD* it is unclear at present if expression is affected by temperature. Furthermore, preliminary experiments with *papG-lacZ* transcriptional fusion in a subclone containing only the *papE, F* and *G* cistrons showed that expression at 25°C was less than half of that at 37°C (unpublished data). Since there may be three different transcriptional units (B-A, C-D and E-F-G;

Norgren *et al.*, 1984, and our unpublished data) we must consider that thermoregulation may act on separate genetic determinants of the *pap* gene system.

One should also consider the possibility that temperature-dependent expression of different virulence factors might be due to a common regulatory system. Two thermoregulatory systems have recently been defined in *E. coli*: the heat shock induction of several polypeptides by the *htpR* (*hin*) gene (Neidhardt and Van Bogelen, 1981; Yamamori and Yura, 1982; Neidhardt *et al.*, 1983); and the *envY* gene regulation of OmpF/OmpC porin production (Lundrigan and Earhart, 1984). However, it is unlikely that these two regulatory systems are involved in the thermoregulation of *pap* genes. The heat shock response is a transient effect within 15–20 min after a temperature up-shift (Lemaux *et al.*, 1978; Yamamori *et al.*, 1978) whereas in the case of *papA* a change to a higher growth temperature resulted in gradual alteration to a new steady-state level of expression (see Figure 2). That the EnvY protein probably does not act in *pap* gene regulation was suggested by the result with the *envY* mutant strain (Table I). In conclusion, expression of the *pap* genes seems to be subject to a novel thermoregulation, and our present findings should facilitate a detailed analysis of the molecular mechanism and identification of regulatory sequences within the *pap* DNA.

Materials and methods

Bacterial strains, plasmids and media

The *E. coli* K-12 strains used were: HB101 (Boyer and Roulland-Dussoix, 1969), MC1061 (Casadaban *et al.*, 1980), RW193 and its *envY* derivative UT4400 (Lundrigan and Earhart, 1984). The Pap$^+$ plasmid pRHU845, the *papB*::Tn*5* mutant pSNO25 and the *papA-lacZ* fusion plasmid pBEU80 (see Figure 1) were described recently (Normark *et al.*, 1983; Norgren *et al.*, 1984). A derivative of pSNO25, plasmid pHMG73, was obtained by an *in vitro* deletion experiment in which all but 50–100 bp of the transposon Tn*5* was removed. The construction of this, presumably non-polar, *papB* mutant, will be presented in detail elsewhere (M.Göransson, unpublished data). The promoter-probing vector plasmid pRZ5202 (L.Munson and W.S.Reznikoff, unpublished data) was used in construction of the *papA-lacZ* transcriptional fusion plasmid pHMG1 (see Figure 1). Plasmid pHMG2 is a derivative of pBR322 (Bolivar *et al.*, 1977) with the *lac* genes transcribed from the tetracycline-resistance gene promoter (see Figure 1). Plasmid pACYC184 (Chang and Cohen, 1978) was used as Pap$^-$ vector control in experiments with pRHU845, and plasmid pSKS106 (Shapira *et al.*, 1983) served as reference for *lacZ* fusion plasmids. Bacteria were grown in Luria-broth and plates contained 1.5% agar. Selection for plasmids was done by adding ampicillin (50 or 100 μg/ml) or tetracycline (15 μg/ml) to the growth media. Lactose MacConkey indicator (Miller, 1972) was used for detection of Lac$^+$ bacteria. Plasmid segregation during growth in an antibiotic-free medium was checked by spreading samples on LA-plates and subsequently testing at least 25 colonies for the plasmid-mediated antibiotic resistance.

Plasmid construction and analysis

Standard cloning methods and agarose gel electrophoretic characterization were used for plasmid constructions (Maniatis *et al.*, 1982). The alkaline extraction procedure (Birnboim and Doly, 1979) was used for screening after transformation. Restriction endonuclease digestions and ligation reactions were carried out under the conditions recommended by the enzyme manufacturers (Boehringer Mannheim GmbH or New England Biolabs Inc.).

Pap antigen assay and hemagglutination tests

Quantitation of Pap pilus antigen was performed by an ELISA as described recently (Normark *et al.*, 1983; Norgren *et al.*, 1984). The ability to cause mannose-resistant hemagglutination (MRHA) of human erythrocytes was monitored by agglutination tests on glass slides as before (Norgren *et al.*, 1984).

Determination of β-galactosidase and β-lactamase

The activity of β-galactosidase was measured as described before (Miller, 1972). A micro-iodometric method was used for determination of the relative amount of β-lactamase in bacterial extracts (Sykes and Nordström, 1972). The Folin-reagent assay was used for protein measurements (Lowry *et al.*, 1951).

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