Growth-rate-dependent synthesis of K99 fimbrial subunits is regulated at the level of transcription

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Increase in the production of the fimbrial adhesin K99 by enterotoxigenic *Escherichia coli* in continuous cultures at specific growth rates above 0.25 h^{-1} was shown to be independent of the nature of the growth-limiting nutrient. The correlation between specific growth rate and K99 production was also found to be independent of the copy number of the K99 operon. Introduction of additional copies of the K99 regulatory region did not affect growth-rate-dependent K99 production in wild-type strains, indicating that no hypothetical regulatory host factor is titrated by the K99 regulatory region. Regulation at the transcriptional level was measured with galactokinase gene fusions. The transcription of the fimbrial subunit gene increased with an increase in specific growth rate. This growth-rate-dependent transcription was found to originate from the strong promoter P_A . Transcription originating from the weaker promoter P_B was independent of growth rate. The results indicated that transcriptional regulation at P_A is involved in the growth-rate-dependent regulation of K99 production.

Introduction

An important step in infections by enterotoxigenic *Escherichia coli* is the adhesion of the bacteria to the intestinal epithelial cells of their hosts. Fimbriae play an important role in this adhesion. K99 fimbriae produced by enterotoxigenic *E. coli* mediate adhesion to bovine, ovine and porcine small intestines (Gaastra & De Graaf, 1982). The available knowledge on the molecular organization of the K99 operon and on the effects of specific growth rate, pH and temperature on the production of K99 fimbriae enabled us to examine the molecular mechanisms underlying physiological regulation.

The eight genes (*fanA–H*) encoding the proteins necessary for expression of K99 fimbriae have been cloned and sequenced (Fig. 1*a*) (De Graaf *et al.*, 1984; Roosendaal & De Graaf, 1989; Roosendaal *et al.*, 1984, 1987*a, b*). They are organized in a single operon. *fanA* and *fanB* encode two regulatory proteins; *fanC* encodes the major fimbrial subunit; *fanD* encodes a polypeptide located in the outer membrane involved in the export of the fimbrial subunits and in the anchorage of the fimbriae in the outer membrane; *fanE* encodes a

periplasmic carrier protein that binds fimbrial subunits and delivers them to the FanD protein; *fanF* to *fanH* encode minor fimbrial components involved in K99 biosynthesis. Analysis of the transcriptional organization of the operon revealed two promoters, P_A and P_B , located upstream from *fanA* and *fanB*, respectively (Roosendaal *et al.*, 1987*a*). Factor-dependent terminators are located between *fanA* and *fanB* (T_1) and between *fanB* and *fanC* (T_2), and a strong rho-independent terminator was found between the structural genes *fanC* and *fanD* (T_3). FanA and FanB have been shown to function *in trans* as anti-terminators of transcription (Roosendaal *et al.*, 1989).

The expression of K99 fimbriae is regulated by environmental conditions. Low temperature, low pH of the medium or the presence of alanine represses the production of K99 fimbriae (De Graaf *et al.*, 1980*a, c*; Girardeau *et al.*, 1982; Van Verseveld *et al.*, 1985). K99 synthesis is also dependent on the specific growth rate. No production takes place at growth rates below 0.25 h^{-1} , but the production increases sharply at higher growth rates (Van Verseveld *et al.*, 1985). The molecular mechanisms involved in the regulation of these phenomena are unknown.

Regulation of K99 synthesis might take place at the transcriptional level, involving the two promoters and/or

Abbreviation: μ , specific growth rate.

Fig. 1. (a) Genetic map of the K99 operon. P, promoter; T, terminator of transcription; A–H, *fanA*–*fanH*; the subunit gene is shaded and dark blocks indicate signal peptides. The arrow indicates the direction of transcription. (b) The regulatory region and the fragments cloned into the *galK* fusion vector for construction of pRR2, pPA and pPB, shown in detail (after Roosendaal *et al.*, 1989).

the terminators, or by post-transcriptional regulation of the gene transcripts. In this study we used gene fusion techniques to investigate whether a transcriptional regulatory mechanism is involved in the growth-rate-dependent control of the production of fimbriae. The transcriptional activity of the two promoters was measured at different growth rates and compared to the transcription of the fimbrial subunit gene. We also examined the effect on production of fimbriae of the presence of multiple copies of the regulatory region.

Methods

Bacterial strains and plasmids. *E. coli* F18, serotype O101:K⁺:K99:F41, was used as the wild-type, K99-producing strain. This strain carries the K99 operon on a low-copy-number conjugative plasmid. *E. coli* strain K12 C600 *galK* was used as a host for the galactokinase fusion plasmids pKO-1, pKG1800 (McKenney *et al.*, 1981), pKC86 (Chak & James, 1985) and their derivatives pPA, pPB and pRR2 (Roosendaal *et al.*, 1989), unless mentioned otherwise. Fig. 1(b) shows the genetic map of the regulatory region of the K99 operon and the fragments cloned into the vector pKO-1 to obtain plasmids pRR2, pPA and pPB (Roosendaal *et al.*, 1989). *E. coli* K12 C600 strain F87 (λ^- , *tonA21 thr-1 leu-6 thi-1 supE44 lacY*), was used as a host for the K99-encoding plasmid pJN99. In the latter plasmid the *Bam*HI fragment of pFK99 containing the K99 operon (Van Embden *et al.*, 1980; De Graaf *et al.*, 1984) has been inserted into the *Bam*HI site of the vector pACYC184.

Growth conditions and media. Strain F18 was grown on minimal medium containing, per litre: KH₂PO₄, 4.5 g; K₂HPO₄·2H₂O, 5.7 g; NH₄Cl, 2.0 g; MgSO₄·7H₂O, 0.2 g; FeSO₄·7H₂O, 5 mg; citric acid, 5 mg; CaCl₂, 11 mg; and glucose monohydrate, 1.98 g, as carbon and energy source for batch cultures and chemostat cultures with glucose as the growth-limiting nutrient. For chemostat cultures with sulphate as growth-limiting nutrient FeSO₄·7H₂O was substituted by 3.6 mg FeCl₂·H₂O, and 5.95 g glucose monohydrate and 18.5 mg MgSO₄·7H₂O per litre were added. For strain F18 transformed with pRR2, ampicillin was added to a final concentration of 200 µg ml⁻¹.

The *galK* strain harbouring derivatives of pKO-1 or pKG1800 was grown in the same medium as above except that only 1 g glucose was added, and the medium was supplemented with 0.5 g yeast extract l⁻¹, 0.5 g Casamino acids l⁻¹ and 300 µg ampicillin ml⁻¹. Strain F87 harbouring pJN99 was cultured as for the *galK* strain except that ampicillin was omitted and chloramphenicol (70 µg ml⁻¹) was added.

Bacteria were grown as continuous cultures in chemostat mode at 37 °C and pH 7.2 (Stouthamer & Bettenhausen, 1975), or as batch cultures on a shaking platform as mentioned. Cultures were in steady state after a minimum of six generation times at constant biomass concentration. Biomass concentrations were measured as described before (Van Verseveld *et al.*, 1985).

Enzyme assays. Samples were taken from steady-state cultures and immediately frozen at –20 °C. Cell extracts for the enzyme assays were prepared by sonicating culture samples for 2 min in 20 s pulses with intermittent cooling. The galactokinase assay was performed as described by McKenney *et al.* (1981). Galactokinase units are expressed as nmol galactose phosphorylated per min per ml of culture with a turbidity (OD₆₆₀) of 1.0. β -Lactamase activity was determined in the same cell extract as galactokinase activity. The enzyme activity was determined in 100 mM-potassium phosphate buffer (pH 7.5) by measuring the difference in absorbance of nitrocefin (compound 87/312) at 486 nm (O'Callaghan *et al.*, 1972). β -Lactamase units are expressed as ΔA_{486} per min per ml culture (OD₆₆₀ of 1.0). Assays were performed in duplicate.

Determination of plasmid concentration. The plasmid content of cells harbouring plasmids with an ampicillin resistance marker was determined by measuring β -lactamase activity as described above. The enzyme activity gives an estimate of the varying content of plasmid at different growth rates (Klotsky & Schwartz, 1987). For determining the content of pJN99, plasmid DNA from a standard amount of cells was isolated by the small-scale extraction procedure of Birnboim and Doly as described by Maniatis *et al.* (1982), using standardized volumes throughout the extraction. Plasmid DNA was loaded onto agarose gels in triplicate and the gels were run according to standard methods (Maniatis *et al.*, 1982). The DNA band intensity was determined by densitometer scanning of photograph negatives of the agarose gels with a laser scanner (LKB Ultrosan 2202). Intensities were taken as a measure of the plasmid content of the culture.

Determination of K99 production. K99 production was determined by enzyme-linked immunosorbent assay (ELISA) of cells resuspended in

phosphate-buffered saline (100 mM-sodium phosphate buffer, pH 7.0, 0.9% NaCl) to an OD₆₆₀ of 0.5 (De Graaf *et al.*, 1980b). The K99 specific antisera and conjugate were a generous gift of F. G. van Zijderveld, Central Veterinary Institute, Lelystad, The Netherlands.

Results

Influence of growth limitation by an anabolic or a catabolic nutrient

The effect of the nature of the growth-limiting nutrient on the regulation of the production of K99 fimbriae was investigated. Previous results have shown that the production of K99 fimbriae takes place at specific growth rates (μ) above approximately 0.25 h⁻¹ and increases with increasing growth rates in continuous cultures with glucose as the growth-limiting nutrient (Van Verseveld *et al.*, 1985). Glucose limitation is of a catabolic nature. The effect of only anabolic limitation was tested by growing the wild-type, K99-producing *E. coli* strain F18 in a continuous culture with sulphate as the growth-limiting nutrient (Fig. 2). Under sulphate-limited conditions K99 fimbriae were produced at μ values above 0.25 h⁻¹, and production increased with increasing values of μ . The production pattern and the level of production were similar to those found for glucose-limited growth. In the experiments described below continuous cultures growing with glucose as the growth-limiting nutrient were used.

Regulation of K99 production in cells with multiple copies of the K99 operon

Previously, it was shown that the production of K99 fimbriae by a K99⁺ wild-type strain increased two- to four-fold after introduction of a multi-copy plasmid containing the K99 operon (Van Embden *et al.*, 1980). This suggests a gene dosage effect that may contribute to growth-rate-dependent K99 production.

The effect of the presence of the K99 operon in multiple copies on growth-rate-dependent synthesis was examined by determining the production of K99 fimbriae by the non-virulent strain F87 containing pJN99, a pACYC184-derived multicopy plasmid harbouring the K99 operon, at different μ values during growth in the chemostat. At $\mu = 0.42$ h⁻¹, production was 6 μ g K99 per ml culture (OD₆₀₀ = 1), while at $\mu = 0.2$ h⁻¹ it was less than 5% of this value. Quantitative plasmid isolation showed that the average plasmid content of the cells at $\mu = 0.42$ h⁻¹ was approximately 50% of that at $\mu = 0.2$ h⁻¹. Thus, production of K99 per operon copy at a high growth rate relative to a lower growth rate is even larger. The results indicated that the

Fig. 2. Production of K99 fimbriae at different specific growth rates by *E. coli* strain F18 in continuous culture with sulphate as the growth-limiting nutrient.

regulatory mechanism involved in growth-rate-dependent K99 production also functioned with the cloned K99 operon, and that this phenomenon was independent of the K99 operon copy number. Also, the regulatory mechanism seems to be present both in the wild-type strain and in the non-virulent strain F87.

Subunit synthesis as a function of the specific growth rate

We aimed to examine the regulatory mechanism underlying the growth-rate-dependent synthesis of K99 fimbriae. Possibly, a limited intracellular concentration of fimbrial subunits decreased production of fimbriae at lower growth rates. Unfortunately, attempts to develop an ELISA specific for single subunits were unsuccessful, probably because the subunit has only one, predominant, antigenic determinant (F. G. van Zijderveld, personal communication).

A different approach was to examine whether the transcription of the operon was growth-rate-dependent. Firstly, we investigated the transcription of the fimbrial subunit gene at different growth rates. In plasmid pRR2, the expression of the *galK* gene is under direct control of the complete K99 regulatory region: promoter P_A, *fanA*, terminator T₁, promoter P_B, *fanB* and terminator T₂ (Fig. 1; Roosendaal *et al.*, 1989). The *galK* gene is fused to the subunit gene (*fanC*) so that the transcription of the *galK* gene, as determined by galactokinase activity, can be taken as a measure of *fanC* gene transcription. Because the fusion vector pKO-1 carries the β -lactamase gene, the activity of this enzyme can be used as an indication of plasmid copy number (Klotzky & Schwartz, 1987). The galactokinase activity was calculated per standard number of *galK* gene copies by dividing the galactokinase activity (U) by β -lactamase activity (U); these values are referred to as relative galactokinase

Fig. 3. K99 production ($\square$) and relative galactokinase activity ($\blacktriangle$) of strain F18 harbouring plasmid pRR2 and grown in continuous culture at different specific growth rates with glucose as growth-limiting nutrient.

activity (AU). The background relative galactokinase activity of the vector without promoter insert (pKC86) in a *galK* strain growing at $\mu = 0.36 \text{ h}^{-1}$ in a chemostat was approximately 11 AU (133 U galactokinase; 12 U β -lactamase).

The wild-type, K99-producing strain F18 was transformed with plasmid pRR2. The background galactokinase activity due to the chromosomal *galK* gene in strain F18 measured at $\mu = 0.5 \text{ h}^{-1}$ was less than 10 U. Values for strain F18(pRR2) were much higher: for example, 460 U of galactokinase and 10.5 U of β -lactamase were measured at $\mu = 0.48 \text{ h}^{-1}$. Subsequently, relative galactokinase activity and K99 production were determined at different μ values with glucose as the growth-limiting nutrient (Fig. 3). The production of K99 fimbriae followed the pattern observed for cultures of strain F18 without pRR2; at μ values above approximately 0.3 h^{-1} an increase in production of fimbriae was observed with increasing μ . Below $\mu = 0.3 \text{ h}^{-1}$ no K99 production could be measured. Furthermore, increasing μ correlated with increasing relative galactokinase activity. This suggested that *in vivo* an increase in transcription of the subunit gene *fanC* will take place at higher growth rates.

The galactokinase activity of pRR2 was also measured in a *galK* strain which produces no K99 fimbriae. The relative galactokinase activity pattern was similar to that obtained in strain F18 (results not shown).

Promoter activity as function of the specific growth rate

The increase in transcription rate of the subunit gene described above could be due to a regulatory mechanism influencing promoter activity at P_A and/or P_B or terminator efficiency at T_1 and/or T_2 , or a combination

Fig. 4. Relative galactokinase activity of the *galK* strain harbouring pPA ($\circ$) or pPB ($\bullet$) and grown at different specific growth rates in continuous culture with glucose as growth-limiting nutrient.

of these effects. Possible regulation of promoter activity was tested by determining the relative galactokinase activity of the *galK* strain transformed by plasmids pPA and pPB (see Fig. 1), at different μ values (Fig. 4). The galactokinase activity controlled by promoter P_B remained constant at approximately 33 AU at different μ values. Plasmid pPA, however, showed an increase in galactokinase activity with increasing μ . This indicated an increase in transcription originating from promoter P_A . Both plasmids gave rise to galactokinase activities above the level resulting from the vector pKC86 without promoter insert (11 AU). All the μ values examined were lower than $0.7 \mu_{\text{max}}$ and no glucose could be detected in supernatant fractions, indicating that the phenomena are not caused by catabolite repression.

Discussion

The regulatory mechanism underlying the previously reported growth-rate-dependent production of K99 fimbriae by enterotoxigenic *E. coli* (Van Verseveld *et al.*, 1985) was examined by measuring K99 production under different growth conditions as well as by determining the transcriptional regulation.

The growth-rate-dependent production of K99 fimbriae was similar with sulphate or glucose as the growth-limiting nutrient, indicating that growth rate regulation is independent of the nature of the growth-limiting nutrient.

E. coli K12 harbouring pJN99 with the cloned K99 operon also showed an increase of K99 production at higher growth rates, indicating that the cloned K99 operon contained all the genetic information necessary for growth-rate dependent production. No change in growth-rate-dependent K99 production was found when the cells contained an excess of the K99 regulatory

region. This suggested that no hypothetical regulatory host factor, either a repressor or an activator of transcription, was titrated by the K99 regulatory region at lower or higher growth rates, respectively.

Transcriptional regulation was examined with galactokinase gene fusions. Expression of *galK* from its own promoter is independent of the growth rate (Duester *et al.*, 1982), indicating that the stability of *galK* mRNA is not affected by the growth rate. Therefore, we assume that the variations in galactokinase activity found in galactokinase gene fusions were a direct reflection of regulatory mechanisms exerted by the DNA fragments inserted in the vector.

The expression of the *galK* gene in pPA increased with increasing growth rate, indicating an increase in transcription originating from promoter P_A . In contrast, the expression of the *galK* gene fused to P_B was constant, indicating a constant activity of promoter P_B . On the basis of our results we cannot identify the mechanism that determines the growth-rate-dependent transcription of P_A . Also, we have no indications that either of the two well-known regulatory factors, ppGpp and cAMP, is involved. GC-rich sequence stretches and a 16 bp stretch between the -10 and -35 region of the promoter have been implicated in growth-rate- and ppGpp-dependent stringent control of transcription (Lindahl & Zengel, 1986; Deneer & Spiegelman, 1987; Baracchini & Bremer, 1988; Dickson *et al.*, 1989). Such sequences are absent around the -35 and -10 region of promoter P_A (Roosendaal *et al.*, 1987a). The nucleotide sequence upstream from P_A shows a high degree of homology with the consensus CAP-binding sequence and catabolite repression has been assumed to be involved in the regulation of K99 production (Ebright *et al.*, 1984; Isaacson, 1980, 1983). Catabolite repression can take place in continuous cultures at high μ values ($>0.7 \mu_{\max}$) (Bull, 1985). Our experimental data, however, indicated that catabolite repression was not involved in the growth-rate-dependent regulation. The transcription of P_A was measured in glucose-limited continuous cultures at μ values that were always lower than $0.7 \mu_{\max}$ and consequently no catabolite repression takes place. Also, the expression of the *galK* gene in plasmid pPA was not affected by addition of external cAMP to a concentration of 5 mM (unpublished results). Furthermore, K99 production by strain F18 was also measured in sulphate-limited continuous cultures with glucose in excess. This supports the idea that the growth rate regulation is not mediated by catabolite repression. Possibly, initiation of transcription at P_A is regulated by the degree of supercoiling of the DNA (Higgins *et al.*, 1988; Dorman *et al.*, 1988; Dimri & Das 1988; Léon *et al.*, 1988) or the stability of mRNA transcribed from P_A is affected by different rates of 5' endonuclease activity, as has been

found to regulate the growth-rate-dependent expression of *ompA* (Melefors & Von Gabain, 1988; Nilsson *et al.*, 1984).

The increase in galactokinase activity by strain F18 harbouring pRR2 could be interpreted as a linear rise as indicated in Fig. 3, but alternatively two levels of activity with a rise at $\mu = 0.2 \text{ h}^{-1}$ and $\mu = 0.5 \text{ h}^{-1}$ might be distinguished. If a linear relation exists between growth rate and transcription, our experimental approach gives rise to a large spread of the measured values (Fig. 3). Similar variations in K99 production levels have been found in different cultures under identical growth conditions (Van Verseveld *et al.*, 1985). A linear increase in transcription of *fanC* as indicated by the galactokinase activity from pRR2 could be accounted for by the increase in transcription of P_A (Fig. 4). Assuming two levels of galactokinase expression from pRR2 implicates a more complicated mechanism of transcriptional regulation.

A remarkable difference was observed between the pattern of increase of transcription of *fanC* and that of K99 production. This indicates that besides a growth-rate-related increase in transcription also a post-transcriptional regulatory mechanism must be functioning. Since all K99 proteins (FanA–FanH) are essential for K99 biosynthesis, any of the many steps involved in this process may be a target for post-transcriptional regulation.

A likely hypothesis could be the growth-rate-related stability of a mRNA species or mRNA secondary structure. In this respect it should be mentioned that the transcript of the intercistronic region between *fanC* and *fanD* might fold into a hypothetical stem and loop structure (terminator T_3) that contains the ribosome-binding site and possible start codon for FanD (Fig. 5). Uncoupling of transcription and translocation at low growth rate (Bremer & Dennis, 1987) may allow the formation of this mRNA secondary structure, while the increase in ribosome concentration and higher rate of polypeptide chain elongation at high growth rate may have the reverse effect. An analogous mechanism of post-translational regulation of growth-rate-dependent gene expression has been described for the *gnd* structural gene of *E. coli* (Carter-Muenchau & Wolf, 1989). Studying the levels and stability of mRNA in chemostat samples may give additional information about the growth-rate-dependent regulation of K99 synthesis.

Baecker *et al.* (1988) found evidence for a third promoter in the K99 gene cluster, located between *fanD* and *fanE*. However, our nucleotide sequence data do not reveal any promoter sequence in this region (Roosendaal & De Graaf, 1989).

Further research will be done to investigate which factors are involved in the transcriptional regulation at

Fig. 5. Secondary structure of mRNA transcribed from the intercistronic region between *fanC* and *fanD* (predicted after Zucker & Stiegler, 1981). The possible ribosome-binding (Shine-Dalgarno) sites (SD, boxes) and matching *fanD* start codons (arrows) are indicated.

P_A and other mechanisms that regulate the synthesis of K99 fimbriae.

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