INFECTION AND IMMUNITY, Apr. 1980, p. 190-194
0019-9567/80/04-0190/05$02.00/0

Vol. 28, No. 1

Factors Affecting Expression of the *Escherichia coli* Pilus K99

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An enzyme-linked antibody centrifuge assay for the detection of *Escherichia coli* pilus K99 was developed and shown to be a specific and quantitative assay for the detection of cell-bound K99. The data presented demonstrate the usefulness of the assay as a diagnostic tool. Using the assay, several factors that affect expression of K99 were investigated. Expression of K99 was dependent upon the degree of aeration provided: nonaerated bacteria produced little or no K99, whereas aerated bacteria produced large amounts of K99. K99 also appeared to be produced only by logarithmically growing cells, whereas there was a demonstrable decline in the amount of K99 per cell during stationary phase. Glucose was shown to repress K99 expression. At 0.5% glucose, K99 expression was highly repressed. Glucose-mediated repression could be overcome by the addition of cyclic adenosine 3',5'-monophosphate. Several other carbon sources also inhibited K99 expression, including pyruvate, arabinose, and lactose; glycerol was stimulatory.

Colonization of the small intestine is a necessary prerequisite for enterotoxigenic *Escherichia coli* (ETEC) to induce diarrheal disease. Colonization occurs by adhesion to the host mucosal surface (2, 3, 8, 17, 20); this adhesion is mediated by specific pili found on the outer surfaces of ETEC (12, 15, 21). One such pilus, K99, which is genetically encoded for on a transmissible plasmid, mediates adhesion of ETEC to the small-intestinal mucosa of neonatal calves, lambs, and pigs (9, 16, 18, 22).

In some experiments with K99 it was demonstrated that the detection of K99 on some K99$^+$ ETEC was variable (6, 11, 13, 18). Thus, Minca media were developed to enhance K99 detectability (6, 7). In one of those experiments it was also shown that glucose repressed the production of K99. The synthesis of several bacterial toxins appears to be repressed by glucose, including the *E. coli* heat-stable enterotoxin (1) and staphylococcal enterotoxins A, B, and C (14). The addition of exogenous cyclic adenosine 3',5'-monophosphate (cAMP) to heat-stable enterotoxin-producing ETEC overcomes the glucose-mediated repression of heat-stable enterotoxin synthesis. Guinée et al. (6) showed, however, that addition of cAMP to K99$^+$ *E. coli* did not derepress glucose-mediated repression of K99.

In addition to glucose-mediated repression, expression and detection of K99 are subject to control by other mechanisms. For example, it was found that passage of aerobically grown ETEC in liquid medium before plating increases the detectability of K99 on K99$^+$ ETEC (11, 13). K99$^+$ strains recently isolated from clinical specimens usually do not appear to produce K99 in vitro; however, after several passages in liquid medium, K99 is detectable. The effect of passage in liquid medium correlates with the emergence of acapsular mutants (13). Presumably the mucoid capsule on some ETEC "masks" K99, and therefore the acapsular mutants are more suitable for K99 detection. Expression of K99 is also dependent upon the temperature at which the cells are grown. Cells grown at 18°C instead of 37°C do not produce K99, nor do they produce other pili (4, 12, 14).

One approach to controlling diarrheal disease induced by ETEC is to interfere with small-intestinal colonization. A further understanding of the mechanisms regulating pilus expression and detection may give insight into how this may be done. In this report is described a quantitative enzyme-linked antibody centrifuge (ELAC) assay for the detection of cell-bound K99. Using this assay, several factors affecting K99 expression were identified; I also observed that expression is indeed subject to classical cAMP-dependent catabolite repression.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* K-12 strain 1475 and its K99-positive transconjugate, 1474 (9), were used in the development of the ELAC assay for the detection of K99 described below and in the studies on K99 expression. Six ETEC isolated from calves with diarrhea (11, 13), four of which were K99 positive and two of which were K99 negative, were used to determine the detectability of K99 on mucoid isolates. Bacterial cells were inoculated 1:20 from a statically

grown culture into Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) and incubated at 37°C with shaking (260 rpm). Unless specified, the TSB contained 0.15% glucose (as supplied by the manufacturer).

K99 antiserum and K99-AP. New Zealand white rabbits were vaccinated twice, subcutaneously, at an interval of 4 weeks with 250 μg of purified K99 (9) in Freund complete adjuvant per vaccination. One week after the second vaccination, the rabbits were bled. The serum was separated and stored frozen at −20°C. Alkaline phosphatase was conjugated to the K99 antiserum with glutaraldehyde by using the procedure of Engvall and Perlman (5), producing anti-K99–alkaline phosphatase (K99-AP).

ELAC assay for K99. Cells to be tested were grown as described, collected by centrifugation at 10,000 × *g* for 10 min at 4°C, and washed three times by repeated suspension in saline (0.85% NaCl) and centrifugation. The washed cell pellet was suspended in saline to a concentration of approximately 10^9 cells per ml. Plate counts were performed to determine the actual concentration of viable cells. A 0.5-ml volume of K99-AP (diluted 1:10,000) was added to 0.25 ml of cells, and the mixtures were incubated for 30 min at 37°C with gentle shaking in glass test tubes. The cells were then collected and washed three times as before. The final pellet was suspended in 0.5 ml of substrate buffer (0.05 M sodium carbonate, 0.01 M $MgCl_2$, pH 9.8), and at timed intervals 0.5 ml of substrate buffer containing *p*-nitrophenyl phosphate (2 mg/ml) was added. After 7 min, the reaction was stopped by addition of 0.1 ml of 1 N NaOH, and the cells were removed by centrifugation. The amount of colored *p*-nitrophenol in the reaction supernatants was measured spectrophotometrically at 400 nm, and the amount was corrected for viable cell concentration. The corrected absorbance at 400 nm is proportional to the amount of K99 per cell and is therefore referred to as the measurement of K99 concentration.

RESULTS

ELAC assay for K99. *E. coli* strains 1474 ($K99^+$) and 1475 were each grown for 18 h and then used in the ELAC assay described in Materials and Methods. The results shown in Fig. 1 were obtained after the addition of various amounts of K99-AP to the cells. *E. coli* 1475, the $K99^-$ parent, did not bind appreciable amounts of K99-AP. Even when 500 μl of K99-AP was added, essentially none was bound to strain 1475. On the other hand, addition of K99-AP to strain 1474 resulted in the formation of detectable antigen-antibody complexes. The addition of increasing amounts of K99-AP to strain 1474 resulted in a proportional increase in the amounts of K99-AP bound to strain 1474. However, when greater than 400 μl of K99-AP was added, a plateau was observed. Additions of K99-AP over 400 μl did not result in increased K99-AP bound to the $K99^+$ strain. In subsequent experiments, it was decided to use 500 μl of K99-AP for standard assay conditions.

Requirement for aeration. *E. coli* 1474 was grown either statically or with vigorous shaking. After incubation for 18 h both strains were tested for K99. The nonshaken culture had an absorbance at 400 nm of 0.110, and the shaken culture had an absorbance at 400 nm of 0.652. Thus, at least a sixfold increase in K99 production occurs in response to mechanical aeration.

Period of K99 production. *E. coli* 1474 was grown for 24 h in TSB. At various times samples were removed, the turbidity was measured spectrophotometrically at 420 nm, and the amount of K99 produced was determined. The results (Fig. 2) demonstrate that a 1-h lag in K99 production occurred, followed by a logarithmic increase similar to the observed increase in cell mass. As the culture approached stationary phase there was a decline in the amount of cell-bound K99. This decline continued for 24 h. K99 did not appear to be secreted into the medium during the decline period. This was determined

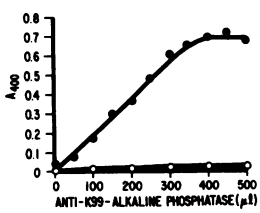

FIG. 1. *ELAC assays of E. coli strains 1474 (●) and 1475 (○). Various amounts of K99-AP (1:10,000) were added to 0.25 ml of washed, concentrated cells that had been grown for 18 h, and the mixture was adjusted to 0.75 ml by addition of sodium chloride (0.85%). Mixtures were then treated as described in the text. Each point represents the mean of three assays.*

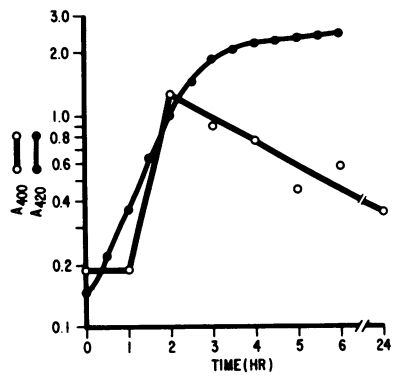

FIG. 2. *Kinetics of K99 production of E. coli 1474. At timed intervals, samples of culture were removed, the turbidity was measured (●) in a spectrophotometer at 420 nm, and K99 was determined (○) by ELAC assay.*

by measuring the amount of cell-free K99 in the culture supernatant by using a sensitive enzyme-linked immunosorbent assay (the procedure will be reported elsewhere). Using this procedure, no cell-free K99 was detected in the culture supernatant. Thus the fate of K99 on stationary-phase cells is unknown.

Glucose effect. It was previously reported that production of K99 was repressed by glucose (6). This phenomenon has now been confirmed by determining the amount of K99 produced when cells were grown for 2.5 h with different concentrations of glucose (Fig. 3). When cells were grown in TSB prepared without glucose, K99 was produced at low levels. At a glucose concentration of 0.15%, maximal K99 production occurred. Increasing the concentration of glucose over 0.15% caused a repression of K99 production. At 0.5% glucose, K99 production was highly repressed. As the cultures grew, there was a measurable decrease in the pH of the medium containing glucose. Even though the decrease in pH was small (about 0.5 pH units), it was possible that the repression of K99 resulted from the production of acids. Therefore, two cultures were grown in 2% glucose (a concentration that represses K99 expression); one was titrated to the starting pH by addition of 0.1 N NaOH at 15-min intervals. The two cultures were compared with a third culture grown in 0.15% glucose. Maintenance of the culture pH did not affect K99 production (Table 1). Both cultures grown in 2% glucose were repressed, whereas the one culture in 0.15% glucose was not.

A more likely explanation for the observed repression of K99 by glucose is that expression is subject to classical cAMP-dependent catabolite repression. Figure 4 shows the results when

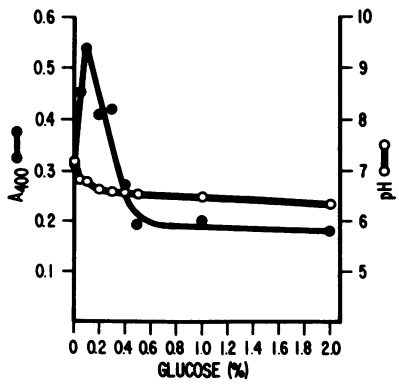

FIG. 3. *Effect of glucose on K99 production and culture pH. E. coli 1474 was grown for 2.5 h in TSB containing various amounts of glucose. The pH of the culture was measured* (○), *and the amount of K99 produced was determined* (●) *by the ELAC assay.*

TABLE 1. *Effect of pH on glucose-mediated repression of K99*

Sample	pH		A_{400}[a]
	Initial	Final	
Glucose (0.15%), not titrated	6.95	6.66	0.680
Glucose (2.0%), not titrated	6.96	6.46	0.247
Glucose (2.0%), titrated	6.96	6.96	0.243

[a] (A_{400}) is proportional to the amount of K99 detected per cell.

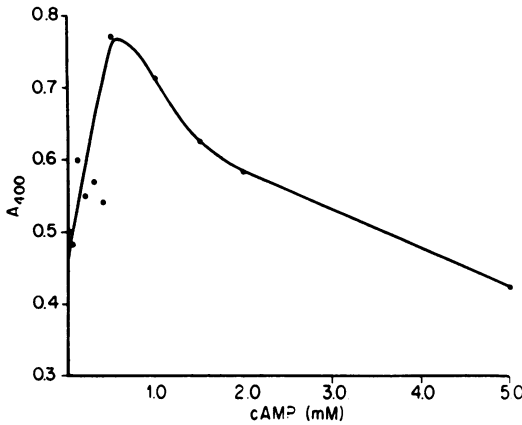

FIG. 4. *Effect of cAMP on K99 production. E. coli 1474 was grown in TSB containing 1% glucose and various amounts of cAMP. After 2.5 h of incubation, the amount of K99 produced was determined by the ELAC assay. The K99-AP used in this experiment had a higher specific activity with respect to alkaline phosphatase than the K99-AP in the previous experiments described in this report.*

E. coli 1474 was grown in TSB containing 1% glucose and various amounts of exogenous cAMP. Addition of cAMP overcame the repression of K99 due to glucose. At a cAMP concentration of 0.5 mM, maximal expression occurred. Increases in the amount of cAMP over 0.5 mM, however, resulted in subsequent decreases in the amount of K99 produced. At a cAMP concentration of 5 mM, there was no apparent effect of the glucose-mediated repression of K99. The growth rates of strain 1474 in the various concentrations of cAMP were the same. This was determined by measurements of turbidity at 420 nm.

A variety of other carbon sources were added to TSB (minus glucose) to determine their effect on K99 production (Table 2). Pyruvate and arabinose were as inhibitory as 1% glucose, whereas lactose was only partially inhibitory. On the other hand, glycerol was highly stimulatory, re-

TABLE 2. *Effect of several carbon sources on K99 expression*

Carbon source (concn)		A_{400}[a]
Glucose	(0.15%)	0.856
Glucose	(1.0%)	0.404
Lactose	(1.0%)	0.552
Glycerol	(1.0%)	1.476
Arabinose	(1.0%)	0.406
Pyruvate	(1.0%)	0.336

[a] Absorbance at 400 nm (A_{400}) is proportional to the amount of K99 detected per cell.

sulting in a 1.7-fold increase in K99 production compared to 0.15% glucose.

Detection of K99 on mucoid *E. coli* isolates. The usefulness of the ELAC assay for the detection of K99 on recently isolated mucoid strains was investigated. Six fresh isolates, four that were shown to produce K99 and two that were shown not to produce K99, were compared with *E. coli* strains 1474 and 1475. Each strain was grown for 2.5 h in TSB and used in the ELAC assay. Cells from the washed pellet obtained during preparation for the assay were also tested for K99 by slide agglutination using K99 antiserum. K99 was detectable on the four mucoid K99$^+$ isolates by the ELAC assay, even though three of four were negative by slide agglutination (Table 3). The amount of K99 detected on the mucoid strains was approximately one-fourth the amount detected on strain 1474. In contrast, no K99 was detected on the two mucoid K99-negative isolates nor on strain 1475. Thus, the assay may be useful in detecting K99 on recently isolated field strains.

TABLE 3. *Detection of K99 on various strains of E. coli by ELAC assay*

Strain	K99		A_{400}[a]
	Genotype[b]	Phenotype[c]	
1474	+	+	0.819
1475	−	−	0.035
3A	+	−	0.207
4B	+	−	0.186
6A	+	+	0.245
14A	+	−	0.185
3E	−	−	0.047
19D	−	−	0.053

[a] Absorbance at 400 nm (A_{400}) is proportioned to the amount of K99 detected per cell.

[b] Genotype was determined previously (11).

[c] Phenotype was determined by slide agglutination using anti-K99 and the concentrated, washed cells used above.

DISCUSSION

The ELAC assay appears to be a sensitive, quantitative, and specific assay for the detection of cell-bound K99. The assay, which is in many respects similar to conventional micro-enzyme-linked immunosorbent assays, is specific for the detection of K99. At a concentration of K99-AP yielding maximum binding to the K99$^+$ strain, essentially no K99-AP was bound to the isogenic K99$^-$ strain. This was anticipated since the antiserum used in the assay was prepared by vaccination of rabbits with purified K99 and therefore should be specific for K99. In a comparison of results from the ELAC assay and slide agglutination it was shown that the ELAC assay detected K99 on K99$^+$ isolates that were negative by slide agglutination. To demonstrate K99 on these strains by slide agglutination, it was first necessary to do three serial passages in TSB (11, 13). Thus, in comparison, the ELAC assay is more sensitive than slide agglutination, and since it can be completed in a few hours versus 4 days it is more rapid. In the diagnostic laboratory, K99 can be used as an indicator of ETEC infections in calves (11, 13), and thus the time saved by using the ELAC assay may be important.

The studies on expression of K99 demonstrated that net production of K99 occurs exclusively during logarithmic phase. The amount of cell-bound K99 declines in stationary phase. Expression of K99 also appears to be related to the degree of aeration and likely the oxygen concentration. In a comparison of aerated and nonaerated cultures, the cells in the aerated flasks produced about six times more K99 per cell than those in the nonaerated flasks. These results are consistent with at least one model of intestinal colonization by K99$^+$ ETEC. The host animal ingests a relatively small dose of ETEC that produce K99. Since the ingested cells are likely to have been in stationary phase, they will possess relatively few K99 pili (that is, they would be relatively nonpiliated). As the bacteria descend the small intestine, those cells that do have pili will adhere to the mucosal epithelium. It is presumed that the oxygen concentration directly adjacent to the epithelium is reasonably aerobic and certainly more aerobic than in the lumen (19). Therefore, those cells growing logarithmically will produce more K99. It is also presumed that the more pili a bacterium has the greater its adhesive capacity. Increased K99 production, therefore, would result in more intensive and extensive colonization of the small intestine by the existing cells and by future daughter cells. Expression of enterotoxin synthesis in vitro also appears to be regulated by the oxygen concentration. Colonization of the small intestine by adhesion to the mucosa would, therefore,

also enhance enterotoxin synthesis, which would result in host diarrhea. As the bacterial cell concentration in the small intestine increases, the bacteria would enter stationary phase. As demonstrated in vitro, there is a decline in the amount of K99 per bacterium in stationary phase. The decrease in K99 would eventually result in a reversal of colonization and the ultimate expulsion of the relatively nonpiliated bacteria into the outside environment.

The experiments reported here confirm the observation made by Guinée et al. (6) that expression of K99 is repressed by glucose. At a glucose concentration of 0.5% or greater, K99 expression is repressed. Other carbon sources, such as pyruvate, arabinose, and lactose, also are inhibitory. In contrast to a previous report (6), however, it was demonstrated that the glucose-mediated repression of K99 expression could be overcome by the addition of cAMP. Maximal derepression was observed when 0.5 mM cAMP was added. At 1.5 mM and 5 mM cAMP, the concentrations used by Guinée et al., little or no derepression occurs. Thus, the reason why cAMP did not in the past derepress glucose-mediated repression of K99 expression was that the concentrations used were not optimal. From the experiments reported here, it is apparent that the expression of K99 is subject to classical cAMP-dependent catabolite repression.

ACKNOWLEDGMENTS

This work was performed with the technical assistance of P. Richter and R. Schneider.

The work was supported by grants from the Horace Rackham Graduate School, The University of Michigan, and from the Public Health Service, National Institute of Allergy and Infectious Diseases, U.S.-Japan Cooperative Medical Program (AI-16191-01), and by a contract from the Public Health Service, National Institute of Allergy and Infectious Diseases (AI-62514).

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