Effect of Glucose and Amino Acids on Expression of K99 Antigen in Escherichia coli

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K99 antigen production by enterotoxigenic Escherichia coli strains of bovine origin was investigated by slide agglutination and in vitro attachment to intestinal villi. Work with two strains (B41 and B44) showed that on minimal medium M2, K99 antigen was not repressed by a high concentration of glucose (2%, w/v). Growth on synthetic or complex medium did not affect K99 antigen detection, which was independent of capsular antigens, and its synthesis was not repressed by Casamino acids or glucose. A survey of 12 strains revealed two groups: in one group K99 antigen production was constitutive on basal medium without glucose, and in the second group K99 antigen was produced only in the presence of glucose. Immunoelectrophoresis patterns, and the results of slide agglutination and attachment tests, were dependent upon K99 type, whereas haemagglutination patterns were not.

INTRODUCTION

Many bovine strains of enterotoxigenic Escherichia coli express K99 antigen, which facilitates the attachment of bacterial cells to intestinal epithelium. This proteinaceous fimbriae-like antigen is plasmid-mediated and is not expressed when the organisms are grown at 18 °C (Sojka, 1971; Smith & Lingood, 1972; Ørskov et al., 1975; Meyers & Guinée, 1976; Morin et al., 1976; Contrepois et al., 1978; Dubourguier et al., 1978). In practice, K99 detection can be included in a serological scheme for the identification of enteropathogenic E. coli (Moon et al., 1976; Guinée et al., 1976; Contrepois et al., 1979). However, routine bacteriological media are not suitable for the detection of K99. This may be due to K99 antigen being masked by capsular K antigen or repressed by a catabolic effect of glucose (Guinée et al., 1977; Isaacsen, 1980).

In order to define the factors influencing K99 synthesis and detection, we studied the influence of the components of Minca medium and different carbon and energy sources on K99 expression.

METHODS

E. coli strains and media. Reference strains B41 (O101:K99), B44 (O9:K30:K99) and K12:K99:Nal' were obtained from Dr R. Sellwood (Institute for Research on Animal Diseases, Compton, Newbury, Berks., U.K.). The other strains, 86a, 85a, 117a (O101:K30:K99), 63a, 13a, 73b (O101:K28:K99), 11a (O101:K41:K99), 92b (O8:K99) and 54b (O9:K99) were isolated from diarrhoeic calves.

All strains were grown for 15 to 24 h at 37 °C on moist slopes or in static broth cultures. The basal medium (BM) was the minimal casein medium (Minca medium) of Guinée et al. (1976), without glucose. Minca medium was supplemented with 1% (v/v) Polyvitex (Bio Merieux) to obtain Minca Polyvitex medium (MPV). The composition of Polyvitex is as follows (final concentration in MPV, mg l⁻¹): vitamin B12, 0.1; L-glutamine, 100; adenine, 10; guanine hydrochloride, 0.3; p-aminobenzoic acid, 0.13; L-cystine, 11; glucose, 1000; NAD, 2.5; cocarboxylase, 1; thiamin hydrochloride, 0.07; cysteine hydrochloride, 250; Fe(N03)3, 0.2.

BM was also supplemented with either Casamino acids or with glucose, both at final concentrations ranging between 1 and 25 g l⁻¹, and sterilized by filtration (pore size, 0.45 μm). BMG2 consisted of BM supplemented with

Abbreviations: CS, K99-constitutive; GD, K99-glucose-dependent; AIE, agarose immunoelectrophoresis; NIE, Noble agar electrophoresis.
2.0 g glucose l⁻¹, and BMG25 was BM supplemented with 25 g glucose l⁻¹. Synthetic medium M2 had the following composition (g l⁻¹): KH₂PO₄, 1.36; Na₂HPO₄, 2H₂O, 10.1; (NH₄)₂SO₄, 2; salt solution, 1 ml. This salt solution contained (g l⁻¹): MgSO₄, 7H₂O, 8; NaCl, 4H₂O, 1; FeCl₃, 6H₂O, 0.135; CaCl₂, 2H₂O, 0.4. The composition of M2 was similar to that of BM except for the nitrogen source which was ammonium sulphate in M2. The carbon substrate was added after sterilization by filtration (pore size, 0.45 µm). The final substrate concentration ranged between 1 and 20 g l⁻¹.

Strains were also grown on a rich complex medium (G20) (Raibaud et al., 1966).

**K99 antiserum.** Sera were produced in sheep and rabbits according to the technique of Sojka (1965) with *E. coli* strains B41, B44, 86a, 63a and K12: K99 : Nal' grown at 37°C. K99-specific antiserum was obtained either by absorbing the K12: K99 antiserum with strain K12 Nal' grown at 37°C or by absorption of the B41 antiserum with strain B41 grown on BM supplemented with 0.2% (w/v) L-alanine at 37°C. L-Alanine specifically inhibits K99 synthesis (Girardeau et al., 1982).

**Slide agglutination, attachment and haemagglutination assays.** Production of K99 was detected by agglutination with specific K99 antiserum (1/10 dilution) and by attachment of the bacterial cells to isolated intestinal villi as previously described (Girardeau, 1980). The maximum attachment index of 100 was obtained when an average of 30 bacteria were attached along a 50 µm length of brush border.

Slide haemagglutination tests were performed as described by Evans et al. (1979), using erythrocytes of the following types: human group A Rh⁺, sheep, calf, guinea pig and horse. The tests were done in the presence of 0.5% (w/v) D-mannose at 4°C and the results scored from − to 4⁺.

**Preparation of partially purified K99 antigen.** K99 antigen was prepared from strains B41, 86a, 54d, 117a, 73b, 63a, 92b and K12: K99 : Nal' grown on BM supplemented with 0.1%(w/v) Casamino acids, BMG2 and BMG25. After culture in Roux flasks (18 h, 37°C), bacteria were harvested in phosphate-buffered saline (PBS) pH 7.2 (containing, g l⁻¹: Na₂HPO₄, 2H₂O, 5.3; KH₂PO₄, 2.7; NaCl, 8.7) and heated at 60°C for 15 min. After centrifugation (27000 g, 10 min), the supernatant fraction was centrifuged at 110000 g for 200 min and sedimented material suspended in 0.1 M-phosphate buffer pH 7.2.

**Immunoelectrophoresis and gel double diffusion techniques.** Immunelectrophoresis was performed in Noble agar (Difco) (Noble agar immunelectrophoresis: NIE) or in agarose standard (Low m, Bio-Rad) (agarose immunelectrophoresis: AIE) according to Scheidegger (1955) with veronal/HCl buffer (0.05 M; pH 8.2) and precipitin arcs were stained with amidol black. Agar double diffusion tests (Ouchterlony) were done in 0.9% (w/v) agarose (Merck) in 0.05 M-veronal/HCl buffer, pH 8.2. As the K99 antigen diffused very slowly, K99 extract as added to the well 8 h before addition of antiserum.

**RESULTS**

**Effect of glucose and Casamino acids on K99 production by E. coli strains B41 and B44 in BM.**

In liquid BM without glucose, increasing the concentration of Casamino acids (1 to 20 g l⁻¹) resulted in a tenfold increase in bacterial counts (4 x 10⁷ cells ml⁻¹ for 2 g l⁻¹ and 4 x 10⁸ cells ml⁻¹ for 20 g l⁻¹). At or above 15 g l⁻¹, the bacterial cells were larger but gave a homogeneous suspension. As assessed by slide agglutination, K99 was synthesized at all concentrations tested, but the maximum attachment index (90) was achieved only when the Casamino acids concentration exceeded 5 g l⁻¹.

In BM, varying the glucose concentration between 1 and 20 g l⁻¹ did not affect the final bacterial numbers after 16 h incubation. At or above 5 g glucose l⁻¹, we observed spontaneous bacterial agglutination, resulting in sedimentation; with 20 g l⁻¹, the supernatant was clear. Capsule stains of strains B41 and B44 showed no significant difference between bacterial cells grown on 1 or 20 g glucose l⁻¹, though adsorption of particles of India ink on the bacterial surface and auto-agglutination of bacterial cells suggested altered surface properties at high glucose concentrations. However, K99, as detected by specific slide agglutination, was synthesized at all concentrations tested. The maximum attachment index was obtained at or above 5 g glucose l⁻¹.

With Casamino acids at 10 g l⁻¹ in liquid BM, the final bacterial counts were still independent of glucose concentration (2 x 10⁹ ml⁻¹). At or above 10 g glucose l⁻¹, the bacterial cells formed aggregates. The cell size was approximately doubled and polymorphism appeared in cultures with 20 g glucose l⁻¹. Many cells were 5 µm in length and their shape varied, some showing cytoplasmic 'vacuoles'. Attachment properties did not change but the maximum attachment index fell to 20 bacteria per 50 µm because of the enlargement of the bacterial cells. Again, formation of K99 was confirmed by specific slide agglutination.

With Casamino acids at 25 g l⁻¹ in addition to glucose at 20 g l⁻¹, K99 synthesis was
Constitutive and glucose-dependent K99 antigen

completely inhibited in liquid BM. When this medium was solidified with agar, growth at the surface allowed K99 synthesis to occur.

After growth on the rich complex medium G20, the bacteria were seen as long (5 μm) cells. The specific K99 agglutination test and attachment test were both negative. After subculture on BM, the strains recovered their original shape.

Production of K99 antigen on minimal medium M2 supplemented with glucose or glycerol

Static broth cultures of strains B41, 86a, 117a and 54d were grown for 16 h at 37 °C in M2 supplemented with either 0.2% (w/v) glucose or 0.2% (w/v) glycerol. These cultures were used as inoculum for cultures in M2 supplemented with either glucose (2 to 20 g l\(^{-1}\)) or glycerol (2 to 20 g l\(^{-1}\)). Irrespective of the strain and serotype used, or the nature of carbon source and its concentration in M2 medium, slide agglutination and in vitro attachment tests showed a similar production of K99 antigen. However, the bacterial growth was fivefold greater on glucose than on glycerol.

Glucose dependence of K99 production

Previous studies had shown that some strains (K12 : K99 : Nal', 63a, 73b, 92b) were K99-positive on MPV medium but were K99-negative on BM, the only difference between these media being the Polyvitex. Glucose (at a final concentration of 1 g l\(^{-1}\) in MPV) was the only component of Polyvitex that affected K99 synthesis. Using BM and BMG2, two groups of strains could be recognized. Those in the first group (B41, B44, 86a, 85a, 117a and 54d) expressed K99 antigen when grown on BM and BMG2, and were termed K99-constitutive (CS). The second group (63a, 13a, 73b, 92b, 11a and K12 : K99 : Nal') produced K99 antigen only when grown on BMG2, not on BM, and were termed K99-glucose-dependent (GD). On synthetic M2 medium, strains of the K99 constitutive group always expressed K99 antigen whatever the carbon source (amino acids, tricarboxylic acids, pentoses or hexoses), whereas those in the glucose-dependent group expressed K99 antigen only with certain carbon sources, such as glycerol, oxalate, tartrate, lactate, glucose, lactose, maltose and fructose, but not with pyruvate, acetate and amino acids.

Characteristics of the K99-constitutive and the K99-glucose-dependent strains

The properties of the two groups of strains are summarized in Table 1. In the CS group, slide agglutination by specific K99 antiserum was positive when strains were cultured with or without glucose. In the GD group, this agglutination was negative for organisms cultured without glucose, but positive after culture on BMG2. The slide agglutination of GD strains was less intense than that with CS strains. Agglutination was incomplete and non-agglutinated cells were apparent in the slide test.

CS strains attached to isolated intestinal villi whatever the culture medium, whereas the GD strains attached only after growth on BMG2. When villi were preserved in buffered formalin for several weeks, as opposed to being kept at −20 °C, attachment of K99+ GD strains was eliminated whereas attachment of K99+ CS strains was reduced but still detectable.

The CS strains B41 and B44 agglutinated the erythrocytes of different species (sheep, calf, guinea pig, horse), when grown in the presence or absence of glucose. On the other hand, GD strains caused haemagglutination only after culture on BMG2. However, the two groups did not share a common haemagglutination pattern. For example, the haemagglutination pattern of GD strains K12 : K99 : Nal' and 92b was similar to that of the CS strains, but differed from the GD strain 63a, which did not haemagglutinate sheep and calf erythrocytes.

Immunoelectrophoresis with the extract from the CS strain B41 tested against specific K99 antiserum showed a K99 cathodic line after growth on BMG2 and BM (Fig. 1, A, B). The cationic precipitin line was susceptible to electroendosmosis in Noble agar (NIE) and stretched towards the cathode. In contrast, extracts of GD strains (63a, 92b, K12 : K99 : Nal') gave no K99 precipitin line if the strains were grown without glucose (Fig. 1, E). After growth in BMG2 medium the NIE pattern showed a cathodic precipitin line shorter than those of CS strains (Fig. 1, D). This line was slightly susceptible to electroendosmosis, its shape being similar on agar or
Table 1. Type of K99 antigen and results of slide agglutination, attachment and haemagglutination tests for E. coli strains

<table>
<thead>
<tr>
<th>K99 type*</th>
<th>Strains</th>
<th>Glucose concn in BM (g 1⁻¹)</th>
<th>Culture temp. (°C)</th>
<th>K99 production</th>
<th>Haemagglutination† with different erythrocyte species:</th>
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<tr>
<td></td>
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<td>K99 slide agglutination by specific K99 antiserum</td>
<td>Attachment index</td>
</tr>
<tr>
<td>CS</td>
<td>B41</td>
<td>0</td>
<td>37</td>
<td>+</td>
<td>100</td>
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<tr>
<td></td>
<td>B44</td>
<td>2</td>
<td>37</td>
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<td>100</td>
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<tr>
<td></td>
<td>86a</td>
<td>2</td>
<td>18</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>54d</td>
<td>2</td>
<td>18</td>
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<td>0</td>
</tr>
<tr>
<td>GD</td>
<td>63a</td>
<td>0</td>
<td>37</td>
<td>-</td>
<td>0</td>
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<td></td>
<td></td>
<td>2</td>
<td>37</td>
<td>+</td>
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<td>2</td>
<td>18</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>92b</td>
<td>0</td>
<td>37</td>
<td>-</td>
<td>0</td>
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<td>2</td>
<td>18</td>
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<td></td>
<td>2</td>
<td>18</td>
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* CS, constitutive; GD, glucose-dependent.
† 4+, haemagglutination instantaneous and complete; lesser degrees of haemagglutination recorded as 3+, 2+, 1+ or negative (−). The haemagglutination pattern results of four tests on different erythrocyte species are shown.

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**Fig. 1.** Noble agar electrophoresis with extracts of E. coli strains B41, K12: K99 : Nal⁺ and 63a. Wells: A, extract of B41 (K99-constitutive) grown on BMG2; B, extract of B41 grown on BM; C, extract of K12 : K99 : Nal⁺ (K99-glucose-dependent) grown on BMG2; D, extract of 63a (K99-glucose-dependent) grown on BMG2; E, extract of 63a grown on BM. Troughs: 1, K99 specific antiserum; 2, non-absorbed 63a antiserum. The K99 cathodic lines from B and D are indicated in the line drawings. Note the non-K99 precipitin line in E.

agarose. With strain K12 : K99 : Nal⁺, the K99 line was longer than with GD strains (63a) but still insensitive to electroendosmosis (Fig. 1, C).

In AIE the electrophoretic pattern obtained with the two groups of strains was similar when the pH of veronal buffer varied from 5.0 to 8.2. Therefore, the difference of shape in precipitin lines between the two groups was not related only to differences of pI. By double diffusion in
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Fig. 2. Agar immunodiffusion of extracts of E. coli strains against non-absorbed B41 antiserum (centre well). The wells contained extracts as follows: 1, B41 (K99-constitutive) grown on BM; 2, B41 grown on BMG2; 3, 54d (K99-constitutive) grown on BM; 4, 54d grown on BMG2; 5, 92b (K99-glucose-dependent) grown on BM; 6, 92b grown on BMG2; 7, 63a (K99-glucose-dependent) grown on BM; 8, 63a grown on BMG2.

agarose, the continuity between K99 precipitin lines of the two groups proved the antigenic identity of the two K99 antigens (Fig. 2).

Glucose dependence among different serotypes of K99 enterotoxigenic E. coli

In 52 serotyped K99+ strains isolated from diarrhoeic calves, 44% were constitutive and 56% were glucose dependent. Among the CS strains, 65% were of serotype O101 and 17% belonged to serotype O9. Serotypes O101 : K28 (22%) and O101 : K30 (26%) predominated in these K99-constitutive strains. Among the K99-glucose-dependent strains, 86% were of serotype O101 and 7% belonged to serotype O8. Serotypes O101 : K28 (48%) and O101 : K41 (28%) were predominant. The capsular K30 antigen was only found among K99-constitutive strains, and K41 only on K99-glucose-dependent strains. Enterotoxicity tests according to Dean et al. (1972) did not show a relationship between K99 type and ST activity.

DISCUSSION

In order to explain the difficulties encountered in detecting K99 after culture on selective media, Guinee et al. (1977) suggested two hypotheses: (1) K99 masking by an abundant synthesis of capsular polysaccharides, or (2) catabolic repression by glucose of enzymes involved in K99 synthesis. Evidence favouring the latter was presented by Isaacson (1980).

In this study, expression of surface properties of E. coli was greatly influenced by the culture medium. The addition of increasing concentrations of glucose and Casamino acids to BM gradually gave a cell morphology similar to that observed after culture on G20 medium. However, there was no increase in capsule thickness as estimated by a capsule stain. On rich medium (BM containing Casamino acids at 25 g l−1 and glucose at 20 g l−1), bacterial cells spontaneously sedimented, which impaired K99 detection by serological agglutination tests.

Previously, the inhibitory effect of L-alanine on K99 biosynthesis was shown (de Graaf et al., 1980b; Girardeau et al., 1982) but it was noted that when L-alanine was below 8 mM in the medium, K99 antigen could be detected by specific agglutination, even in rich media which gave large amounts of capsular antigen as evidenced by mucoid colonies. In addition, we observed no connection between K99 detection and the lack of K(A) antigens (strains B41, 92b, 54d) or the presence of various K antigens (strains 86a, B44, 63a).

K99 synthesis in synthetic M2 medium showed no catabolite repression by glucose, even with
The results of slide agglutination tests always correlated with haemagglutination tests, in vitro attachment and formulation of precipitin lines in immunoelectrophoresis. However, the haemagglutination patterns obtained with erythrocytes of different species were not dependent upon K99 type: the haemagglutination patterns of K99-positive strains probably reflected the presence of an anionic antigen (F41) present on some K99 strains (Morris et al., 1980).

Although the immunoelectrophoresis patterns were dependent upon K99 type, the shape of the precipitin lines, their mobility and especially their sensitivity to electroendosmosis suggested differences in electrophoretic properties not related to the pI values of the antigen. Moreover, the expression of K99 in GD strains depended on the carbon source used. Thus, aspartate or pyruvate allowed good bacterial growth on M2 but did not lead to K99 synthesis, whereas oxalate or tartrate allowed K99 synthesis even when growth was slight.

The redox potential is also an important factor. A previous report (Girardeau et al., 1982) showed a greater inhibitory effect of l-alanine when organisms were grown under anaerobic rather than aerobic conditions. In the same manner, the glucose-dependent expression of K99 is sensitive to redox potential. This in vitro effect of redox potential seems to be paradoxical. Generally, the intestine is thought to have a low redox potential. This suggests either the existence of particular growth conditions in the intestine, which allow K99 synthesis even at low redox potential, or the presence of a higher redox potential in the region of the epithelial cells.

The significance of constitutive and glucose dependent K99 production in vivo is unknown. Studies of 52 K99-positive E. coli strains, isolated from diarrhoeic calves, showed that 23 were of the constitutive type and 29 of the glucose-dependent type, but results of enterotoxicity tests according to the technique of Dean et al. (1972) were unrelated to K99 type.

De Graaf et al. (1980a) and Renault et al. (1980) demonstrated a relationship between K99 expression and O serotype. A similar relationship appears between the K99 type and serotypes. For example, the frequency of O101 : K28 is twofold higher in GD strains than in CS strains. The serotype O101 : K30 is found only with CS strains and O101 : K41 is found only with GD strains. Finally, strain K12 : K99 : Na+, obtained by transfer of the constitutive K99 plasmid from B41, showed an unusual K99 antigen. Its immunoelectrophoresis pattern resembled the CS type, but the synthesis of K99 was glucose dependent. Therefore, the expression of structural genes may depend on the energy metabolism of the host cell (K99 synthesis requires particular carbon sources) and K99 assembly may depend on enzymes involved in envelope synthesis. These hypotheses need to be tested further.

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