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## EXPRESSION OF K99 PILUS OF *E. COLI*

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### Abstract

Different factors are involved in production and (or) detection of K99. They are modifications in plasmid contents of the strains, the size of capsular antigens, the quantity of K99 protein produced according to the culture media. We made a distinction between the strains which produced K99 on Minca medium without glucose (K99-C) and those which needed glucose for production on this medium (K99-GD). The K99-GD strains were able to use adonitol and most K99-C strains did not. With strains K99-C we showed that heavily capsulated colonies produced as much K99 than non capsulated colonies, but heavily capsulated ones were hardly detected with slide agglutination. According to the carbon substrate, differences were observed in K99 production. Mannose was a bad carbon source for K99 production, the synthesis of which was completely inhibited with K99-GD strains, but not with K99-C strains. Adonitol was a good substrate for detection of K99 from K99-GD strains. Mesure of intracellular AMPc showed that there was non catabolite repression for K99 biosynthesis. In the opposite, high intracellular level of AMPc was correlated with a low production of K99. Plasmid contents of the strains indicated that most strains had one or two plasmids in the range of 50-70 megadaltons. Small differences in plasmids are sometimes correlated with modifications in the quantity of K99 produced. The main conclusions were that the distinctions that we proposed between K99-C and K99-GD could help understand the mechanisms of K99 production. Large size of the capsules and a low level of K99 production both contributed to a bad detection of K99 with slide agglutination. There was no catabolite repression for K99 biosynthesis. Some small modifications in the size of the plasmids were sometimes correlated with differences in quantitative production of K99. These differences among K99 *E. coli* probably explain why some K99 *E. coli* diarrhea escape to diagnostic. A synthetic medium with glucose and adonitol is perhaps able to improve detection of K99 *E. coli* from fecal samples.

The production and/or detection of K99 are (is) stable feature(s) in some strains (reference strain B41, for example) but not in most wild strains. Three main parameters can explain this variability. The first is a loss of the plasmid or some molecular modifications which alter gene functions. A second, already noted by Ørskov *et al.* (1975) and Guinée *et al.* (1977) is the presence of capsular antigens. A third is the composition of culture media. The last led Guinée *et al.* (1977) to propose the Minca medium which improves K99 detection. Others results indicate a peculiar effect of L-alanine (Girardeau *et al.*, 1980, 1982a; de Graaf *et al.*, 1981) which completely inhibits K99 synthesis. According to Isaacson (1980), glucose has a catabolic-repressing effect on the biosynthe-

sis of K99. However, glucose increases it and is even necessary on a Minca base with some *E. coli* strains, according to Girardeau *et al.* (1982b). These conflicting results on the glucose effect show the complexity of the problem and the need for a careful study of different K99 *E. coli*.

From the results established by Girardeau *et al.* (1982a) which differentiate strains where K99 biosynthesis is glucose-dependent (K99-GD) on a Minca medium, from others where it is constitutive (K99-C) phenotypic features of both strain groups were considered, and the effects of capsular antigen and different culture conditions were studied. The possible effect of intracellular AMPc will then be considered and the plasmid content of

a few strains will be estimated. Finally, some practical implications of our observations for diagnoses will be discussed.

## Materials and Methods

### *Bacterial strains*

K99 *E. coli* strains were isolated from diarrheic calf feces in France from 1978-1979. Some serotypes were established by Dr Renault (1980) and others by Dr Ørskov of the International Escherichia and Klebsiella Centre.

### *K99 Antiserum*

A specific K99 antiserum was prepared according to Girardeau *et al.* (1982a).

### *Visualisation of capsule*

Observed with a light microscope according to the classical wet India ink film method.

### *Culture media*

Bacteria were grown on a Minca medium (Guinée *et al.*, 1977) either with glucose (MG+) or without (MG-).

A minimal medium was made with the mineral base of a Minca medium and 2g/l of  $\text{SO}_4(\text{NH}_4)_2$  as a nitrogen source instead of casamino acids. The carbon substrate was either glucose (m Glu), mannose (m Man), adonitol (m Ado), dulcitol (m Dul), raffinose (m Raf), sucrose (m Suc), sorbose (m Sorb), or rhamnose (m Rha). Sugars were added to the medium as a concentrated filter-sterilized solution (0.22  $\mu$ ) for a final concentration of 2 g/l.

### *K99 production measurement*

The ELISA method as described by Girardeau *et al.* (1982a). A semi-quantitative K99 measurement was obtained by SDS-PAGE intensity of the spots. Extraction of pili was always performed with the same amount of bacteria obtained on an agar medium and suspended in 1 ml of saline ( $1.8 \times 10^{10}$  *E. coli* — about 50-70 mg dry weight of bacteria according to the strain). Extraction and gel electrophoresis were carried out according to Girardeau *et al.* (1982a). Protein bands were silver-stained according to Oakley *et al.* (1981). With this procedure, spots on the gel corresponded to  $3 \times 10^6$  bacteria (about 75  $\mu\text{g}$  dry weight of bacteria).

### *AMPc measurement*

Performed according to a method described by Joseph *et al.* (1982).

With this indirect method, AMPc was measured

in the culture medium. The synthesis rate of AMPc (P moles d'AMPc/mg dry weight of bacteria) produced in the culture medium in the early exponential growth curve, correlated with the intracellular AMPc level (Joseph *et al.*, 1982). AMPc was determined according to a radio-immunological method with the Institut Pasteur Production Kit (code 79 830).

### *Isolation of plasmids and agarose DNA electrophoresis*

Carried out according to Kado and Liu (1981).

## Results

### *Phenotypes of the K99-C and K99-GD E. coli (table 1)*

There is no particular distinction between K99 positive *E. coli* and K99 negative *E. coli* except that 70% of K99 positive *E. coli* are adonitol<sup>+</sup>. Among K99 negative *E. coli*, this feature is only 5 to 6% according to Edwards and Ewing (1972). The utilisation of adonitol differentiates K99-GD strains from K99-C strains.

All K99-GD strains are adonitol<sup>+</sup> and most K99-C strains are adonitol<sup>-</sup>. The few exceptions for K99-C *E. coli* are two O101:K28 strains and, the reference strain B41.

### *Production of capsules among K99<sup>+</sup> E. coli*

Dissemination of our K99<sup>+</sup> strains onto a minimal medium with glucose yielded some clones of heavily capsulated bacteria and others with non-capsulated bacteria. A comparative study of K99-C and K99-GD strains (table 2) showed that a dissociation between heavily-capsulated and non-capsulated clones was obtained only with K99-C strains of serotype O101:K30, O9:K(A) and O?:K(A).

The K99 slide agglutination of capsulated bacteria was very weak compared to the strong agglutination of the non-capsulated bacteria. K99-GD strains did not dissociate like K99-C strains. Large colonies with heavily-capsulated bacteria which produced a weak agglutination in K99 specific antiserum were always noted.

### *Effects of the composition of culture media and capsules on K99 production*

A semi-quantitative estimation of K99 production was performed after K99 extraction and gel electrophoresis by evaluating the intensity of K99 bands on gel. The culture media were chosen according to previous results obtained through

Table 1. — Phenotypic features of *E. coli* K99 positive strains.  
(footnotes, see page 403).

Group Serotype	(No of strains)	Drug resistance (No of strains)						Colicin No of strains			
		Ap	Sm	Km	Cm	Tc	Su	+	-	Nd <sup>a</sup>	
<b>K99 GC</b>											
O?:K(A) <sup>-</sup>	(3)	S <sup>b</sup>	R <sup>b</sup>	S	R	R	R	(1)	1	...	2
		S	S	S	S	S	S	(1)			
		R	R	R	R	R	R	(1)			
O101:K(A) <sup>-</sup>	(3)	R	R	R	R	R	R	(2)	...	1	2
		S	R	S	S	R	R	(1)			
O101:K28	(2)	R	R	S	R	R	R	(1)	1	...	1
O101:K30	(5)	R	R	R	R	R	R	(5)	4	...	1
		R	R	S	R	R	R	(1)	1	2	1
O8:K85ab	(1)	S	R	R	R	R	R	(1)	...	1	...
<b>K99 GD</b>											
O101:K(A)	(2)	R	R	R	R	R	R	(2)	...	1	1
O101:K28	(10)	R	R	R	R	R	R	(9)	...	6	4
		S	R	S	S	S	R	(1)	...	6	4
O101:K44	(1)	R	R	R	R	R	R	(1)	1	...	...
O101:K32	(6)	R	R	R	R	R	R	(1)	...	3	3
		S	R	Not done		R	R	(2)			
O8:K85ab	(1)	S	R	R	R	R	R	(1)	...	1	...
<b>API 50 CH characters<sup>c</sup></b>											
Group	(No of strains)	Commun				Variable					
		+	-	+	-						
K99 GC	(18)	30		24, 26, 27, 37, 34		8,4	14				
						14,1	17				
						25,1	17				
						45,4	14				
						46,1	17				
						47,4	14				
K99 GD	(20)	8		46		14,1	19				
						24,1	19				
						25,2	18				
						26,1	19				
						27,1	19				
						30,3	17				
						31,1	19				
						34,1	19				
						35,3	17				
						47,1	18				

Table 2. — Effects of capsule on slide agglutination.

Group Serotype (No of strains)		Size of the capsule	Slide agglutination with a specific K99 antiserum
<b>K99 GC</b>			
O9:K(A)	(2)	colonies with a thick capsular material (white colonies)	negative or weakly positive agglutination
O101:K30	(4)		
O?:K(A)	(1)	colonies with little or no capsular material (bluish colonies)	strongly positive agglutination
<b>K99 GD</b>			
O101:K32	(2)	always very thick capsular material and no bluish colonies	weakly positive agglutination when grown on Minca medium
O101:K28	(4)		
O101:K44	(1)		

slide agglutination with a specific K99 antiserum.

Results (table 3, fig. 1) indicate that the same quantity of K99 was produced by K99-C strains whether capsulated or not. For these K99-C strains, K99 was produced with the four culture media but the MG<sup>-</sup> medium was unfavorable to K99 production and the m-Man medium, moderately favorable. The quantitative production of K99 was:

$$m \text{ Glu} = \text{MG}^+ \geq m \text{ Man} \geq \text{MG}^-.$$

For K99-GD, strains which were all heavily capsulated we studied clones which had been previously selected as good or low K99 producers. With these K99-GD strains, the effects of culture medium was very great. There was no K99 synthesis on MG<sup>-</sup> as already described by Girardeau *et al.* (1982) nor was there any K99 synthesis with mannose. Adonitol was the best carbon source for K99 production with these strains. The quantitative production of K99 with strain 32a<sub>3</sub> was:

$$m \text{ Ado} > m \text{ Glu} > \text{MG}^+ \\ \text{and } \text{MG}^- = m \text{ Man} = \text{O}.$$

With clones of strains 11a and 32a which had been selected as good or low K99 producers with slide agglutination, we confirmed this observation

with gel electrophoresis on m Glu medium (11a<sub>2</sub>  $\geq$  11a<sub>4</sub> and 32a<sub>3</sub>  $\geq$  32a<sub>1</sub>). For the low K99 producers 11a<sub>4</sub> and 32a<sub>1</sub> only the m Ado medium allowed positive slide agglutination with a specific K99 antiserum. Results obtained with strains from our collection, were also compared with strains from other origins, serotypes and biotypes (table 4) to see whether our results were valid. Among these five strains, a K99-GD phenotype could be recognized for strains H416, B117, G559 and B80 according to slide agglutination after growth on the MG<sup>-</sup> medium, but these K99-GD phenotypes did not correlate with growth on adonitol for strain G559 and B80. Strain O64 had a K99-C phenotype. Some of the latter strains could utilize sugars which were not among our collection of strains. A few carbon substrate effects on K99 biosynthesis were then detected as well.

#### *Intracellular AMPc level and K99 production*

Measurement of the AMPc intracellular level and K99 production (table 5) showed that a high AMPc intracellular level correlated with a low K99 production (strain B41 with medium m Man) or no production at all (strain 32a<sub>3</sub> with medium m Man and MG<sup>-</sup>). However, a comparison of results

a : ND: not done

b : S: sensible, R: resistant

c : the code numbers are those of API 50 CH  
common characters for all the strains are:

+ 1, 4, 5, 6, 10, 11, 12, 13, 15, 18, 19, 22, 28, 29, 32, 42, 43

- 2, 3, 7, 9, 16, 17, 20, 21, 23, 33, 35, 36, 37, 38, 39, 40, 41, 44, 48, 49.

Table 3. Production of K99 on different culture media.

Phenotype Serotype Strains	Capsule	Production of K99 according to gel electrophoresis (1) and intensity of seroagglutination (2) after growth on					
		MG <sup>+</sup>	MG <sup>-</sup>	mGlu	mMan	mAdo	
<b>K99 GC</b>							
O101:K30 117 A1	+++	1	A <sup>a</sup> +++	B +	C +++	D ++	NG
		2	+	+	+	-	
117 A3	±	1	E +++	F +++	G +	H ++	NG
		2	+++	+++	+++	++	
O9:K(A) 128 C1	+++	1	ND	ND	I ++	ND	NG
		2	-	-	+	+++	
128 C3	±	1	ND	ND	J ++	ND	NG
		2	+++	+++	+++	+++	
<b>K99 GD</b>							
O101:K28 32 A3	+++	1	K +	L -	M-P ++	N -	O +++
		2	±	-	+	-	++
32 A1	+++	1	ND	ND	Q ±	ND	ND
		2	-	-	-	-	+
O101:K32 11 A2	+++	1	ND	ND	R ++	ND	ND
		2	-	-	+	-	++
11 A4	+++	1	ND	ND	S +	ND	ND
		2	-	-	-	-	+

a: see figure 1.

obtained with media m Ado and m Gly showed that a correlation between intracellular AMPc and K99 production was not absolute. Low or no K99 production on the m Man medium was also associated with slow bacterial growth, but such a correlation was not observed when the rates of exponential growth ( $\mu$ ) with MG<sup>+</sup> and MG<sup>-</sup> were compared.

#### Plasmid contents of some K99 E. Coli

All strains, except for the H 416 had one or two plasmids in the range of 50-70 megadaltons (fig. 2) which is the size of most K99 plasmids already described. The results pertaining to strain H 416 still need further confirmation. K99-C cannot be differentiated from K99-GD strains on

the basis of these results. A comparison of the plasmid contents of strains 32a and 11a with their two good and low K99 producer variants showed different plasmid contents. The good K99 producer 32a3 had only a 67 Mdalton plasmid, compared to the low one 32a1 with two plasmids (67 Mdal and 58 Mdal). Both clones 11a2 and 11a4 had a 55 Mdal plasmid but the good K99 producer 11a2 had a 58 Mdal compared to a 61 Mdal for the low K99 producer 11a4.

#### Discussion — Conclusion

The different parameters for K99 detection and/or production already mentioned by several

authors, all have a role, but with varying degrees according to the strains.

The two large groups K99-C and K99-GD proposed by Girardeau *et al.* (1982) also strongly correlate with the utilization of adonitol with K99-GD strains and non-utilization with the K99-C strains. In other respects, adonitol is a good sub-

strate for K99 production with the K99-GD strains.

The effect of culture medium is extremely pronounced for the K99-GD strains which did not produce K99 on media MG<sup>-</sup> and m Man. This effect is less pronounced with K99-C strains. K99 production is also lower on media MG<sup>-</sup> and

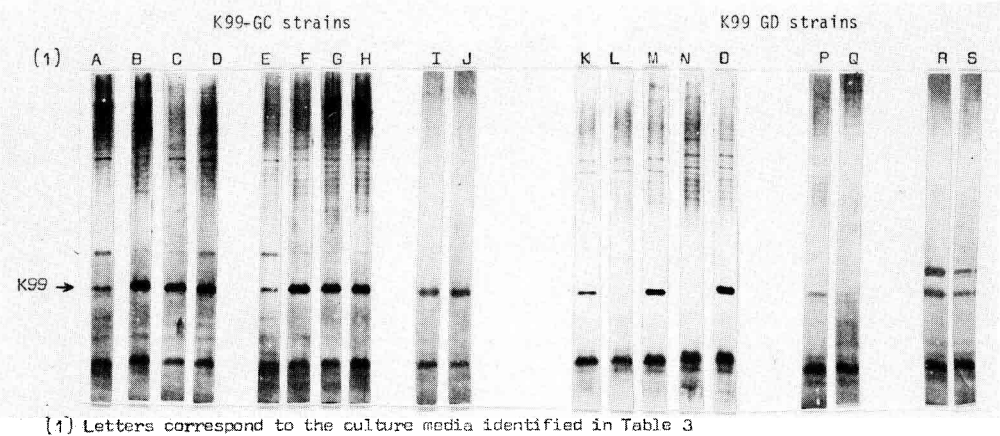


Fig. 1. — Semi-quantitative measurement of K99 production using gel electrophoresis.

PHENOTYPE	K99-C										K99-GD				
	117a		122cc		B41	094	#99	0999	32a		11a	3117	92b	H416	
SEROTYPE	0101:K30		09:K(A)		0101:KA-	0140:147	009:K17	00:K25	0101:K28		0101:K32	09:K25ab	09:K25ab	0101:KA	
COLONIES	117 <sub>1</sub>	117a <sub>3</sub>	122c <sub>1</sub>	122c <sub>3</sub>	0101:KA-				32a <sub>1</sub>	32a <sub>3</sub>	11a <sub>2</sub>	11a <sub>4</sub>			
Plasmids (Megadaltons)	$57 \pm 1$		$67 \pm 1$		$61 \pm 2$		$\approx 61$	$\approx 61$	$\approx 61$	$67 \pm 1$	$67 \pm 1$	$58 \pm 1$	$61 \pm 1$	$\approx 61$	$61 \pm 2$
	$54 \pm 2$		$54 \pm 2$		$54 \pm 2$				$\approx 50$	$58 \pm 1$		$55 \pm 1$	$\approx 55$		
					$3,4$				$\approx 4$	$5,4$	$5,4$		$\approx 11,5$	$9 \pm 1$	$14 \pm 1$
			$2,6$		$2,6$				$\approx 3,7$				$\approx 7$	$6,8 \pm 0,1$	$6,1 \pm 0,1$
					$2,4 \pm 0,1$				$\approx 2,2$				$\approx 3,4$	$3,4$	

Fig. 2. — Plasmids of different K99 *E. coli*.

Table 4. — K99 slide agglutination for different strains on several culture media.

Strains		Culture media									
		MG <sup>+</sup>	MG <sup>-</sup>	mADO	mMAN	mDUL	mSORB	mRAF	mSACCH	mRHA	Phenotype
H416 <sup>a</sup>	0101:K(A)	+	-	++	-	NG	NG	NG	NG	+	K99 GD
B117 <sup>b</sup>	08:K85ab	+	-	++	-	NG	NG	NG	-	±	K99 GD
G559 <sup>b</sup>	08:K25	+	-	NG <sup>c</sup>	+	±	-	±	++	+	K99 GD
B80 <sup>b</sup>	020:K17	+	-	NG	-	NG	+	NG	NG	++	K99 GD
064 <sup>b</sup>	064:K"V14Z"	+	+	NG	++	NG	NG	NG	NG	++	K99 C

a : strains obtained from Dr. Guinée (Netherlands)

b : strains obtained from Dr. Morris (Weybridge)

c : NG: No growth.

Table 5. — Intracellular AMPc and K99 production.

	Culture media					
	MG <sup>+</sup>	MG <sup>-</sup>	mGlu	mGly	mAdo	mMan
<i>E. coli</i> B41, K99 GC						
AMPc (p moles/mg dry weight of bacteria)						
μ (hour <sup>-1</sup> )	1.17	576	144	307	391	1 030
K99 (ELISA titers)	1.39	1.39	1.13	0.87	1.02	0.47
	256	128	64	8	128	8
<i>E. coli</i> 32a3, K99 GD						
AMPc (p moles/mg dry weight of bacteria)						
μ (hour <sup>-1</sup> )	86	290	65	175	160	500
K99 (ELISA titers)	1.30	1.25	1.39	1.02	1.18	0.71
	32	0	128	0	32	0

m Man. The results in table 4 indicate that other carbon substrates are also able to modify K99 production.

In order to explain the effect of the carbon substrate, we studied the validity of the glucose catabolic repression mentioned by Isaacson (1980) but which seemed contradictory with that of Girardeau *et al.* (1982b). An indirect measurement of intracellular AMPc level on different culture media showed that the lowest AMPc intracellular levels, those of a classical glucose catabolic repression, are also those with a more abundant K99 production. The opposite is obtained when the intracellular AMPc level is high. This correlation is particularly evident when comparing the MG<sup>+</sup> to MG<sup>-</sup> and mGlu to mMan media. Although our results corroborated with the intracellular AMPc level they do not lead to the conclusion that the intracellular AMPc level is directly

implicated in K99 biosynthesis regulation. Further results are needed.

In addition, a correlation between slow growth and low K99 biosynthesis which could be explained by a low metabolic activity, as seen with the m Man medium compared to the mGlu medium, is not a completely suitable criterion. It is not true when comparing media MG<sup>+</sup> and MG<sup>-</sup> where growth curves are nearly the same but K99 production is different.

The effect of a large capsule as often seen with K99 *E. coli* is clearly demonstrated with few K99-C strains. Clones heavily or non-capsulated from the same strain produce the same amount of K99 protein but the capsule inhibits slide agglutination with K99-specific antiserum. A large capsule associated with a low K99 production can explain the false negative results of slide agglutination.

Plasmid contents of K99 strains are rather hete-



rogenous. However, most of them appear to have plasmids in the range of 50-70 M dal. The best known K99 plasmid obtained from the reference strain B41 is a 52 M dal plasmid (So *et al.*, 1976). Interestingly, the main observation on these results involves the differences between the plasmid contents of clones obtained from the same strain but which were also differentiated beforehand through a K99 production criterion.

One question remains as regards the detection of K99 *E. coli* according to the classical procedure with slide agglutination after growth on a Minca medium (Guinée *et al.*, 1980).

Two observations indicate that some K99 *E. coli* colibacillosis can escape diagnosis. They were obtained with strains carrying K99 and the F(y) pilus (Girardeau *et al.*, 1980; Contrepolis *et al.*, 1982). With a one-week-old calf, the fecal excretion of an orally administered K99, Fy *E. coli* was studied and in the feces a mixture of (K99<sup>+</sup>, Fy<sup>+</sup>) and (K99<sup>-</sup>, Fy<sup>+</sup>) clones were found through slide agglutination after dissemination on MG<sup>+</sup> or m Glu media. A later study of these (K99<sup>-</sup>, Fy<sup>+</sup>) clones showed that these strains always had the same plasmid content and that K99 production was detectable after extraction and gel electrophoresis or double immunodiffusion. The last observation was based on a 3-day-old diarrheic

calf. Its feces were disseminated on a Minca medium and yielded (K99<sup>-</sup>, Fy<sup>+</sup>) colonies through slide agglutination. When these (K99<sup>-</sup>, Fy<sup>+</sup>) strains were studied, a K99 positive slide agglutination was obtained after growth on mAdo medium.

From a practical point of view, some K99-GD strains are apparently not detected after growth on a Minca medium. It could be interesting to compare a minimal medium with adonitol to a Minca medium in order to improve the detection of some low K99 producers.

The *in vitro* results cannot be extrapolated to the *in vivo* behaviour of the strains. However, such differences could exist for K99 *E. coli in vivo*. At the present time, the possible correlation between strain virulence and the quantitative production of the K99 protein still requires further work. It would also be interesting to evaluate the distinction between the K99-C and K99-GD strains according to our criteria, in other serotypes. Such a classification could lead to a better understanding of the organization and the working of genes coding for K99 biosynthesis.

*EEC seminar on gastro-intestinal diseases in the young pig and calf* 1-3 December 1982, INRA-CRZV de Theix 63110 Beaumont, France.

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