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Plasmids Coding for Enterotoxins, K88 Antigen and Colicins in Porcine *Escherichia coli* Strains of O-group 149

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Abstract. This study was carried out to determine whether the strong epidemiological correlation observed in Sweden between production of the adhesin K88, the heat-stable (ST) and the heat-labile (LT) enterotoxins in *E. coli* strains of O-group 149 isolated from piglet diarrhea might be explained by linkage of their genetic determinants. From 22 different isolates plasmids coding for these virulence factors were investigated by conjugation and transduction experiments and analysis on agarose gels. The genes coding for ST production could be transferred by selection for antibiotic resistance, but behaved as transposable elements most often residing on a 55 Mdal plasmid coding for colicin B. The genes coding for raffinose fermentation and K88 antigen production were located on a 45 Mdal plasmid and the genes coding for LT production on plasmids within the 45–70 Mdal size. Thus the epidemiological importance and spread of this O-group in Sweden was explained by its stable content of two or three virulence plasmids, which could be transferred independently of one another.

Introduction

Escherichia coli of O-group 149 is the most important O-group causing neonatal piglet diarrhea in Sweden (Söderlind and Möllby 1978). Strains belonging to this O-group mostly produce both heat-labile (LT) and heat-stable (ST) enterotoxin, as well as the adhesin K88 (Söderlind and Möllby 1979). The genetic determinants for these virulence factors are mainly plasmid borne. Different plasmids coding for K88 (Bak et al. 1972; Mooi et al. 1979; Shipley et al. 1977), ST and LT (Gyles et al. 1974; So et al. 1975; So et al. 1978) have been described and characterized. Since no enterotoxigenic strains which produced LT only had been found, the ST and LT toxins were for a long time considered to be coded for on the same conjugative plasmid in porcine strains producing both toxins (Gyles et al. 1974; Smith and Gyles 1970). However, strains producing only

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ST were described by Smith and Gyles (1970), and later, both human and porcine strains producing only LT were found (Evans et al. 1977; Isaacson and Moon 1978; Olsson and Söderlind 1980). Recently, however, several reports indicate that at least in porcine strains, LT only strains are often ST^+ when these are investigated in the piglet loop assay (Burgess et al. 1978; Gyles 1979; Olsson and Söderlind 1980). In *E. coli* O 78 strains of human origin LT and ST production were encoded by two separate plasmids (Smith et al. 1979). The genes coding for ST production are probably located on a transposable DNA segment (So et al. 1979) and accordingly determinants coding for ST have been found in plasmids encoding LT (Gyles et al. 1974), CFA I (Smith et al. 1979), CFA II (Penaranda et al. 1980), and possibly K99 (Isaacson and Moon 1978).

The aim of the present study was to determine whether the strong epidemiological correlation between production of LT, ST, and K88-antigen in porcine *E. coli* strains of O-group 149 might be explained by linkage of their genetic determinants. However, the results indicate that these virulence factors are encoded by separate plasmids and that the determinants coding for ST in these strains also behave as transposable elements.

Materials and Methods

Bacterial Strains. Twenty-two *E. coli* strains of O-group 149 were obtained from piglets with diarrhea occurring in different herds in Sweden (Söderlind and Möllby 1978). These were examined with regard to K88 antigen, LT, and ST production, antibiotic resistance pattern and biochemical properties (Table 1). These strains were used as donor strains in this investigation.

As the recipient strain for plasmids from the O 149 strains, an *E. coli* C strain, LH 1035 (F^-his-4 metE-4 rha-1 Raf^--nal^R rough) was used (Hedén and Rutberg 1976), a mutant of strain C-1467 (Wiman et al. 1970). This strain was kindly provided by Dr. L. Hedén, Department of Microbiology, University of Stockholm.

Biotyping, Colicin Typing and Determinations of Antibiotic Resistance. The donor strains were tested for fermentation of rhamnose, lactose, maltose, sucrose, raffinose, xylose, trehalose, arabinose, adonitol, glycerol, sorbitol, dulcitol, salicin and for urea hydrolysis, utilization of citrate and production of acid from mucate. The tests were performed as described by Söderlind (1971).

Production of colicin was determined by the agar overlay method according to Frédéricq (1957). The colicinogenic (Col^+) strains were kindly typed by Prof. P. Frédéricq, l'Institut National de Médicine Vetérinaire, Liège, Belgium.

The minimum inhibitory concentrations (MIC) of antibiotics for wild type and transconjugant strains were determined by the agar dilution method as recommended by Eriksson and Sherris (1971). Strains with a MIC > 50μg for streptomycin, neomycin, ampicillin, tetracyline, chloramphenicol and a MIC > 1000μg for sulpha were regarded as resistant.

Enterotoxin Testing. ST production was determined by the infant mouse method (Dean et al. 1972) and LT production by the adrenal cell test (Söderlind and Möllby 1978).

Table 1. Properties of *E. coli* O 149 strains isolated from piglets with diarrhea

Strain	Enterotoxigenicity ST	LT	K88 Raf	Colicins	Antibiotic[a] resistance pattern
Bd 91/75	–	+	+	–	Su
B 362/75	+	+	+	B	Su, Sm, Tc
B 364/75	+	+	+	B+E	Tc
B 444/75	+	+	+	–	Su, Sm, Tc
Bd 549/75	–	+	+	–	Su, Sm, Tc
Bd 574/75	+	+	+	B	Su, Sm
B 630/75	+	+	+	B	Su, Sm
Bd 852/75	+	+	+	B+Ib	Su, Sm, Tc
Bd 899/75	+	+	+	B	Su, Sm
Bd 1148/75	+	+	+	B	Su, Sm, Tc
Bd 1233/75	+	+	+	B	Su, Sm
Bd 1495/75	+	+	+	B	Su, Sm
Bd 1674/75	+	+	+	B+Ia	Su, Sm
Bd 1693/75	+	+	+	B	Su, Sm
Bd 1876/75	+	+	+	B	Su, Sm
Bd 1877/75	+	+	+	B	Su, Sm, Tc
Bd 2211/75	+	+	+	B	Su, Sm
Bd 2222/75	+	+	+	B	Su, Sm
Bd 2412/75	+	+	+	B+E	Ne
Bd 2443/75	–	+	+	E	Su, Sm
Bd 2450/75	+	+	+	–	Su, Sm
Bd 3835/75	–	+	+	B+Ia	Cm, Su, Sm, Tc, Ap

[a]Abbreviations used for antibiotic resistance: Su = sulpha, Sm = streptomycin, Tc = tetracycline, Ap = ampicillin, Ne = neomycin, Cm = chloramphenicol

Transfer of Plasmids Encoding Raffinose Fermentation and Antibiotic Resistance. Twenty-four hour crosses between donor and recipient strains were performed in nutrient broth with equal volumes of donor and recipient strains, with subsequent selection for nalidixic acid resistance (50μg/ml) and the appropriate antibiotics (50μg/ml) on minimal medium (Meynell and Meynell 1970). In the same way crosses were performed with selection for raffinose fermentation as single carbon source (1%) and nalidixic acid resistance (50μg/ml) on minimal medium. The use of raffinose fermentation as a selective marker in transconjugants depended on the fact that the genes encoding K88 antigen and raffinose fermentation (Raf) are often located on the same non-conjugative plasmid (Smith and Parsell 1975; Shipley et al. 1977).

Transduction of the Raf Determinants. Transduction experiments, using phage P1 kc grown on Raf^+ $K88^+$ ST^+ Col B^+ LH 1035 strains, were essentially as described by Lennox (1955). Transduced LH 1035 bacteria were selected for raffinose fermentation on minimal agar plates as described above.

Analysis of Plasmid Content on Agarose Gels. Partially purified plasmid DNA from certain strains was prepared and run on 0.7% agarose vertical gels for 6 to 8 h as

described by Meyers et al. (1976). The molecular weights of the plasmids were calculated in relation to the mobility of the following reference plasmids: pBR 322 (2.6 Mdal), SA (23 Mdal), RP4 (34 Mdal), and R1 (62 Mdal) (16). After electrophoresis the gels were stained with ethidium bromide (1 ug/ml) and photographed under UV illumination. The reference plasmids were kindly provided by Dr. S. Falkow, School of Medicine. Seattle, Wa., USA.

Results

Biochemical Properties of E. coli O 149 Strains. All O 149 strains listed in Table 1 were very uniform in their biochemical properties and belonged to the same biotype as did more than 90% of all O 149 strains investigated (Franklin, unpublished data). The donor strains fermented mannose, lactose, maltose, raffinose, adonitol, trehalose, xylose, arabinose and sorbitol. They were unable to ferment glycerol, sucrose, dulcitol, and salicin and to hydrolyse urea, or to utilize citrate and to produce acid from mucate.

Colicinogeny. Of the 22 donor strains 18 (82%) were colicinogenic (Col^+) , 17 of which (77%) produced colicin B. Three strains produced colicin I in addition (Table 1).

Transfer of the ST and LT Determinants. In order to obtain LT^+ and ST^+ transconjugants the Su Sm drug resistance determinants of the Su Sm^R donor strains were transferred to LH 1035. The transfer frequency ranged between 2.5×10^{-3} and 1.0×10^{-5} per donor cell except for the strain Bd 3835, where the Su Sm Tc Ap Cm antibiotic resistance markers were transferred concurrently at a frequency of about 1.0×10^{-2}. Ten antibiotic resistant transconjugant clones in each cross were investigated for LT production in the adrenal cell test. In no case were transconjugants, producing detectable amounts of LT found.

Co-transfer of ST and drug resistance determinants was investigated in transconjugants derived from the crosses with two of the Col B^+ donor strains or one Col B^- donor strain. There was frequent co-transfer of determinants coding for Su Sm resistance, ST and Col B production (Table 2). In the mating regarding the Col B^- ST^+ donor strain, only one of ten transconjugants tested was ST^+ (Table 2). None of the antibiotic resistant transconjugants had become Raf^+, $K88^+$, or LT^+. Thus, there seemed to be a close association between the determinants coding for ST and Col B production. It was assumed that the Col B plasmid acted as the transfer factor for the

Table 2. Relationship between Col B and ST production in Su Sm^R transconjugants in matings between O 149 strains and LH 1035

Donor Su Sm^R strains and phenotypes	No of Su Sm^R transconjugant strains with indicated phenotype				Total number of transconjugants investigated
	Col B^+ ST^+	Col B^+ ST^-	Col B^- ST^+	Col^- ST^-	
Bd 899 Col B^+ ST^+	6	0	0	0	6
Bd 1877 Col B^+ ST^+	6	1	1	1	9
Bd 2450 Col B^- ST^+	0	0	1	9	10

ST genes and the Su Sm plasmids (Frydman and Meynell 1969). If this was so, Col B^+ recipient clones would be expected to occur at a certain frequency in crosses between Col B^+ donor strains and LH 1035 with selection for only nalidixic acid resistance (Anderson 1965). In a cross in this vein with the Col B^+ donor Bd 1148 four out of fifty recipient clones were Col B^+. Those Col B^+ clones were also ST^+ but not Su Sm^R, a fact in line with the hypothesis of a common plasmid for determinants encoding ST and Col B production.

To determine whether or not the determinants for ST and K88 production, in the same way as the ST and CFA I (Smith et al. 1979) or CFA II determinants (Penaranda et al. 1980) were linked to each other and whether one could obtain LT^+ transconjugants, the following experiments were carried out. All 22 donor strains listed in Table 1 were mated with LH 1035 and transconjugants were selected on minimal agar for raffinose fermentation. Raf^+ transconjugants appeared at a low frequency in 17 of the 22 crosses (Table 3). All Raf^+ transconjugants tested were agglutinated by K88 antiserum. Fifty clones of Raf^+ transconjugants from each mating were then tested

Table 3. Transfer frequencies of the Raf K88 plasmid and co-transfer frequencies of the LT, ST, and Col B genes for *E. coli* 0 149 strains to LH 1035

Strain	Transfer frequency of the Raf K88 plasmid per donor	No. of Raf^+ clones with the indicated phenotype out of the number of investigated transconjugant clones				
		LT^+	ST^+ColB^+	ST^+Col^-	ST^-Col^-	$SuSm^R$
Bd 91	7.0×10^{-5}	12/50	NA[d]	NA[d]	NA[d]	45/50
Bd 362[a]	3.0×10^{-6}	0/50				
B 364	C[c]	–				
B 444	C[c]	–				
B 549	3.0×10^{-4}	14/50	NA[d]	NA[d]	NA[d]	50/50
Bd 574[a]	2.5×10^{-5}	0/50				8/9
B 630	7.0×10^{-5}	2/50	4/5		1/5	5/5
Bd 852[a]	2.0×10^{-6}	0/50				5/5
Bd 899	1.0×10^{-4}	3/50	9/12		3/12	50/50
Bd 1148	2.8×10^{-4}	3/50	7/10		3/10	7/50
Bd 1233	1.0×10^{-5}	0/50	5/5			
Bd 1495[a]	1.0×10^{-6}	0/50				5/5
Bd 1674	2.1×10^{-5}	tox[b]	10/10			50/50
Bd 1693	3.0×10^{-4}	3/50	9/10		1/10	46/50
Bd 1876[a]	7.0×10^{-6}	0/50				44/50
Bd 1877	2.2×10^{-5}	2/50	10/10			29/50
Bd 2211[a]	2.0×10^{-4}	2/50				45/50
Bd 2222[a]	2.5×10^{-5}	3/50				14/50
Bd 2412	C[c]	–				
Bd 2443	C[c]	–				
Bd 2450	2.3×10^{-5}	12/50	NA[e]	5/15	NA[e]	13/13
Bd 3835	C[c]	–				

[a] ST not tested in transconjugant sublines
[b] Toxic reaction of the adrenal cells
[c] No transconjugants obtained
[d] Not applicable, ST^- strains
[e] Not applicable, Col B^- strains

for LT prodcution. In most matings, 0–6% of the transconjugant bacteria were LT^+ (Table 3). However, in the crosses with two Col^- ST^- LT^+ strains, namely B 549 and Bd 91 and one Col^- ST^+ LT^+ donor strain, namely Bd 2450, about 25% of the Raf^+ $K88^+$ transconjugants were LT^+ (Table 3). Co-transfer of ST determinants with the Raf K88 plasmid was very common, except for strain Bd 2450, and all investigated ST^+ transconjugants with Col^+ donors wer Col^+, whereas the ST^- transconjugants were Col^- (Table 3).

In analogy with the results above ST determinants were also cotransferred with the Raf and K88 determinants from the Col^- donor Bd 2450, though at a reduced rate compared to the Col B^+ donor strains. The transconjugants which were ST^+ in this cross were LT^- and the LT^+ transconjugants were ST^-. Apparantly no linkage existed between the LT and ST determinants in Bd 2450.

Transduction of the ST Gene with the Raf K88 Plasmid. Phage P1 kc was propagated on 20 transconjugant LH 1035 Raf^+ $K88^+$ ST^+ Col B^+ sublines, containing plasmids originating from the donor strains Bd 1148, Bd 1877, Bd 1693, and Bd 899 and transduced to LH 1035.

Raf^+ transductants appeared at a frequency of about 6×10^{-7} on all the minimal agar plates. Of the transductant clones 20 were screened for co-transduction of genes coding for K88, ST and Col B production. All clones tested were $K88^+$ and one out of five containing genes derived from Bd 1148 was ST^+ in addition. All were Col^-. Hence, ST genes can be located on the Raf K88 plasmid.

Plasmid Content of Wild Type, Transconjugant, and Transductant Strains. The plasmid content of some wild type strains, transconjugant and transductant sublines are listed in Table 4. In the Raf^+ $K88^+$ transconjugants and transductants a plasmid of about 45 Mdal mol wt was always present and some Raf^+ $K88^+$ Col B^- ST^- transconjugants contained an additional 40 Mdal plasmid. This 40 Mdal plasmid band was still visible in the transconjugants cured of known determinants. In the Raf^- $K88^-$ Col B^+ ST^+

Table 4 Plasmid content of wild type 0 149 *E. coli* strains and LH 1035 sublines[a]

Donor strains	Plasmid content of donor strains (mol wt in Mdal)	Plasmid content of LH 1035 transconjugant and transductant sublines of indicated phenotypes (Mol wt in Mdal)			
		$Raf^+K88^+ColB^+ST^+$	Raf^+K88^+	$ColB^+ST^+$	$Raf^-K88^-Col^-ST^-LT^-$
Bd 852	80, 62, 40–45	80, 62, 40–45[c]			40
Bd 899	55, 45	55, 45		55	
Bd 1148	45	45[b]	45	45	
Bd 1233	60, 50, 40				40
Bd 1693	70, 55, 45	55, 45[b]	45	55	
Bd 1877	62, 45, 40, 20	45, 40	45		
Bd 2211	50, 40, 20	50, 40			

[a]Plasmids of mol wt < 20 Mdal not shown
[b]No additional band was seen in the LT^+ transconjugants
[c]This subline was $Raf^+K88^+ColB^+ColI^+LT^+$

transconjugants a single plasmid of about 55 Mdal was present. The amount of partially purified DNA in the plasmid preparations from LT^+ LH 1035 sublines was perhaps too low to permit visualization of the LT plasmid in the gels, since production of LT was rapidly lost in the LT^+ transconjugants. It is also possible that the LT plasmid band was obscured by other plasmid bands, representing plasmids of approximately the same size (Table 4). Some wild type strains, however, contained plasmids of about 60–70 Mdal which were missing in the LT^- transconjugant clones and hence probably coded for LT (Fig. 1). All donor and LH 1035 Sm^R strains contained plasmids of about 9 and 4–5 Mdal presumably encoding Su Sm resistance.

Spontaneous Curing of LH 1035 Transconjugants. All transferred plasmids were spontaneously cured in the LH 1035 transconjugant and transductant clones at a high frequency. Even the Raf and K88 determinants segregated, so that some clones became only Raf^+. No strains became $K88^+$ only, which is in agreement with observations by Shipley et al (1977). ST production was lost though the bacteria were still Col^+.

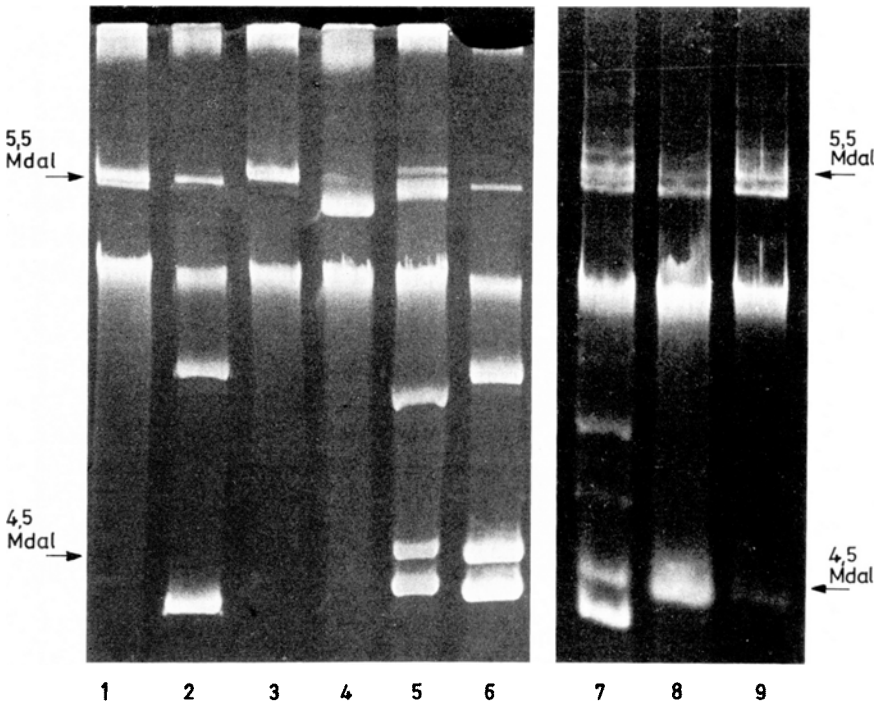

Fig 1. Agarose gel electrophoresis of partially purified plasmid DNA of one wild type strain and different *E. coli* C sublines. Track *1*: $Raf^+K88^+Col\ B^+ST^+$ transconjugant (Plasmid origin Bd 899). Track *2*: Col $B^+ST^+SuSm^R$ transconjugant (Plasmid origin Bd 899). Track *3*: Reference plasmid R1. Track *4*: Reference plasmid RP4. Track *5*: $Raf^+K88^+Col\ B^+ST^+LT^+SuSm^R$ wild type strain Bd 1693. Track *6*: Col $B^+ST^+SuSm^R$ transconjugant (Plasmid origin Bd 1693). Track *7*: Wild type strain Bd 1693. Track *8*: $Raf^+K88^+Col\ B^+ST^+SuSm^R$ transconjugant (Plasmid origin Bd 1693). Track *9*: $Raf^+K88^+Col\ B^+ST^+LT^+$ transconjugant (Plasmid origin Bd 1693)

The inhibition zones around the Col$^+$ colonies, however, gradually decreased in size and perhaps the amount of ST toxin but not of colicin was too low to be detected in the assays used, though the only transconjugant sublines that were stable ST producers were Col B$^+$ too.

Discussion

The epidemic spread of strains of O-group 149 makes it probable that these strains isolated from all over Sweden represent the spread of a single clone (Söderlind and Möllby 1978; Söderlind and Möllby 1979). The uniform biochemical properties and the relatively uniform plasmid content of the strains investigated in the present study support this hypothesis. It was therefore of interest to more closely examine the virulence plasmids of this important enteropathogen.

The present findings demonstrated that the ST and LT determinants resided in different plasmids which segregated in genetic crosses with *E. coli* C. The transmissible nature of the LT plasmid was more difficult to demonstrate in Col B$^+$ donor strains than in the Col$^-$ strains. Hence, the Col B plasmid probably interfered with the transfer of the LT plasmid.

The ST and Col B determinants were most frequently located on the same plasmid as indicated by the results of the mating experiments (Tables 2 and 3) and the appearance of a common single band in the agarose gels. The ST genes may also reside on the K88 plasmid and on plasmids without currently known markers. Thus, the ST determinants derived from individual 0 149 strains may be located on different plasmids. These results are in line with the concept, that the ST determinants behave as transposable elements. They are not in line with the concept that the ST and LT determinants always reside on the same plasmid in porcine LT$^+$ST$^+$ *E. coli* strains (Gyles et al 1974). However, this concept was based on assay of ST production in pigs. As the present findings were based on assay of ST production in infant mice and in view of the recent findings that porcine strains designated LT 'only' not infrequently are ST$^+$ (ST pig$^+$) when investigated in piglet intestinal loops (Burgess et al. 1978; Gyles 1979; Olsson and Söderlind 1980), the LT$^+$ ST$^-$ donor strains and transconjugant sublines derived from them are presently investigated with regard to ST pig (Franklin and Möllby in preparation). Furthermore, the observed co-transfer of antibiotic resistance plasmids and of the plasmids mediating ST production (Tables 2 and 3) suggests a possible enhancement of enteropathogenic virulence of porcine *E. coli* due to antibiotic selective pressure.

In conclusion, the determinants coding for LT, ST, and K88 antigen reside on different plasmids in the 0 149 strains. Thus, the epidemiological importance and spread of this 0-group in Sweden may be explained by its stable content of two or three virulence plasmids, which can be transferred independently of one another.

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