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DNA sequencing of a pathogenicity-related plasmid of an avian septicemic *Escherichia coli* strain

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ABSTRACT. A 43-MDa conjugative plasmid isolated from an avian septicemic *Escherichia coli* (APEC) strain possessing genes related to the adhesion and invasion capacities of *in vitro*-cultured cells was sequenced. The results demonstrated that the 43-MDa plasmid harbors bacterial pathogenicity-related sequences which probably allow the wild-type pathogenic strain to adhere to and invade tissues and to cause septicemia in poultry. The existence of homology sequences to sequences belonging to other human pathogenic Enterobacteriaceae like *Escherichia coli* O157:H7, *Shigella* and *Salmonella* was also observed. The presence of these sequences in this plasmid could indicate that there is horizontal genetic transfer between bacterial strains isolated from different host species. In conclusion, the present study suggests that APEC strains harbor high-molecular weight plasmids that present pathogenicity-related sequences and that these are probably responsible for the

pathogenicity exhibited by these strains. The presence of human pathogenicity-associated sequences in APEC conjugative plasmids suggests that these strains could represent a zoonotic risk.

Key words: Avian, *Escherichia coli,* Plasmids, Adhesion

INTRODUCTION

Escherichia coli causes a variety of diseases in poultry, including respiratory tract infection, septicemia, omphalitis, swollen-head syndrome, enteritis, and cellulitis, which are responsible for significant economic losses in the chicken industry. These strains are denominated avian pathogenic *E. coli* strains (APEC) (Dho-Moulin and Fairbrother, 1999). The pathogenesis and role of virulence factors present in these strains have not yet been fully elucidated, although considerable progress has been made in recent years to establish the mechanisms of pathogenesis (reviewed by Dho-Moulin and Fairbrother, 1999 and by La Ragione and Woodward, 2002). Epithelial adherence and invasion, flagella, toxins and cytotoxins, serum resistance, colicin production, outer membrane proteins, iron sequestering systems, and temperature-sensitive hemagglutinin are virulence factors that have been described for APEC strains (Dho-Moulin and Fairbrother, 1999; Schouler et al., 2004; Tivendale et al., 2004; Amabile de Campos et al., 2005).

Several studies have identified genes encoding virulence factors of APEC. Gene inactivation experiments confirmed a role in the pathogenicity of APEC for type 1 and P fimbrial adhesins (La Ragione et al., 2000a,b), for aerobactin iron transport system (Lafont et al., 1987), and for the temperature-sensitive hemagglutinin Tsh (Provence and Curtiss III, 1994; Dozois et al., 2000). Genome analyses have led to a better understanding of APEC pathogenicity. Selective capture of transcribed sequence studies conducted by Dozois et al. (2003), identified pathogen-specific transcripts in an APEC strain corresponding to putative adhesins, lipopolysaccharide core synthesis, iron transport systems, plasmid and phage encoded genes, and genes of unknown function. Genomic subtraction studies (Schouler et al., 2004) performed among the APEC strain MT512 and the non-pathogenic *E. coli* strain EC79 have identified protein sequence homologues to *Haemophilus influenza, Listeria, Salmonella typhimurium*, EHEC and phage sequences, and homology to pathogenicity island sequences.

The occurrence of plasmid-encoded genes among APEC strains (Dozois et al., 2003) and the presence of virulence genes in plasmids (Johnson et al., 2002; Stehling et al., 2003a,b) demonstrated the role of these genetic mobile elements in APEC pathogenicity. Recently, Stehling et al. (2003a) described a 43-MDa conjugative plasmid (named E plasmid) from a septicemic APEC strain (SEPT13) that transferred to a non-pathogenic and non-invasive *E. coli* strain (MS101) adhesion and invasion capacities. The purpose of the present study was to accomplish the partial DNA sequencing of plasmid 43 MDa (named to date E plasmid) originally found in the septicemic SEPT13 strain and to describe genes that could be related to the pathogenic process present in this strain.

MATERIAL AND METHODS

Bacterial strains and media

Recombinant strain TE was obtained by conjugation assays between an avian septicemic *E. coli* resistant to Amp, Tc and Sm (SEPT13) and an *E. coli* K12 strain (MS101) (non-pathogenic, nalidixic acid resistant) (Stehling et al., 2003b). This strain harbors a plasmid of 43 MDa and is able to adhere to and invade Hep-2 cells and to adhere to trachea epithelial cells. The capacity of this plasmid to be transferred between strains and to be responsible by the pathogenic characteristics was determined by transposon mutagenesis (Stehling et al., 2003b). Plasmid pRA1 (86 MDa) and 8 plasmids harbored by strain V517 (32; 5.12; 3.48; 3.03; 2.24; 1.69; 1.51; 1.25 MDa) (Macrina et al., 1978) were used as molecular standards by electrophoresis on agarose gels. All strains were stored at -80°C in LB medium (Sambrook et al., 1989) containing 15% glycerol to avoid plasmid losses. All media used were those described by Sambrook et al. (1989).

Plasmid DNA extraction and purification

Plasmid DNA extraction of strain TE was performed by the alkaline lysis method (large scale) as described by Sambrook et al. (1989). The plasmid of 43 MDa was directly purified from agarose gels (low-melting point) using β-agarase (Amersham Bioscience) as described by the manufacturer.

Plasmid library construction

Purified plasmidial DNA was randomly fragmented as described by Birren et al. (1997). The fragments obtained were cloned using the TOPO Shotgun Subcloning Kit in the pCR 4 Blunt (Invitrogen) vector, and the products obtained from ligation were electrotransformed into *Escherichia coli* TPO10 (TOPO Shotgun Subcloning Kit - Invitrogen). White transformant colonies were selected on LB-agar medium supplemented with ampicillin (100 μg/mL) and X-Gal (40 μg/mL).

DNA sequencing and analysis

DNA cloned fragments were sequenced by using the Dye Terminator Cycle Sequencing Kit for MegaBACE and M13 forward and reverse primers (Invitrogen). The samples were read in an automatic DNA sequencer (MegaBACE 1000 - Amersham Bioscience). For DNA sequencing analysis, the Phred Phrad software and the basic local alignment search tool software (BLAST) were used (Altschul et al., 1997).

RESULTS AND DISCUSSION

Many plasmid-encoded gene products are required for full expression of virulence in several enteropathogenic bacteria, including those of the genera *Shigella* (Sasakawa et al., 1992), *Yersinia* (Ferber and Brubaker, 1981), *Salmonella* (Kawahara et al., 1988), and *Esche-*

richia coli (Nataro and Kaper, 1998), as well as APEC strains which harbor plasmid-encoded virulence factors. Stehling et al. (2003a) described a plasmid from a septicemic APEC strain that mediated the adhesion and invasion phenotypes for Hep-2 cells as well as adherence to epithelial tracheal cells. Using conjugation assays, a 43-MDa plasmid (named E plasmid) from the septicemic APEC SEPT13 strain was transferred to a non-plasmid harboring, non-pathogenic MS101 *E. coli*. The recombinant strain (named TE strain) was able to adhere to and invade Hep-2 cells and to adhere to tracheal epithelial cells. To better characterize and identify possible virulence factors and pathogenicity-related genes present on plasmid E we accomplished the DNA sequencing of this genetic mobile element.

Twenty-three open-reading frames (ORFs) were identified in plasmid E-DNA sequencing analysis (Table 1). BLAST analysis showed that among these ORFs, eighteen showed over 90% similarity to *E. coli*, *Shigella sonnei*, and *S. flexneri* genes.

Table 1. Open-reading frames (ORFs) identified in plasmid E of the APEC strain TE.

ORF	Homology found by BLAST	Gene/protein	Organism	Similarity (%)	Access number
1	ORF f276	H	*E. coli*	100	AAA97221.1
2	ORF 4	*rep*A	*E. coli*	100	AAA26064.1
3	ORF 1	Rrf	*S. sonnei*	98	AAB39946.1
4	ORF f147	H	*E. coli*	97	AAA58189.1
5	ORF 163	*tag*A	pO157 (*E. coli*)	96	CAC05847.1
6	ORF B	IS150	*E. coli*	97	AAB18535.2
7	ORF	H	*S. flexneri*	97	AAN42144.1
8	ORF F	TraV/R	*E. coli*	97	AAB61939.1
9	ORF 161a	U	*S. flexneri*	97	CAC05845.1
10	ORF 63	U	*E. coli*	96	AAD47185.1
11	ORF 169	U	*E. coli*	94	AAA99217.1
12	ORF	*finO*	pR100 (*E. coli*)	94	CAA28566.1
13	ORF 73	U	Plasmid F	94	AAD47179.1
14	ORF	H	O157:H7 (*E. coli*)	93	AAG57625.1
15	ORF 160	U	*S. flexneri*	92	CAC05844.1
16	ORF 159b	YccB	*S. flexneri*	91	CAC05843.1
17	ORF 197	TraX	*S. flexneri*	90	CAC05864.1
18	ORF	TraI	*E. coli*	90	CAA39338.1
19	ORF 52	EAF	*E. coli*	82	BAA84887.1
20	ORF 161b	*yce*A	*S. flexneri*	76	CAC05846.1
21	ORF 29	p50Hb	*S. enterica*	75	BAB20536.1
22	ORF J	*Tra* U	*E. coli*	73	AAB61938.1
23	ORF 58	FocD	*E. coli*	26	CAA21381.1

Several ORFs showing high similarity to those described to be present in the Enterobacteriaceae were detected (Table 1). Among these ORFs with high similarity to those described in virulence-related plasmid were ORFs 9, 15, 16, 17 and 20 (Table 1) which showed over 90% similarity to ORFs 161a (unknown gene), 160 (unknown gene), 159b (protein YccB),

and 197 (gene *Tra*X), and 76% similarity to 161b (gene *yce*A), respectively. These ORFs were described on plasmid pWR100 from *S. flexneri* (Buchrieser et al., 2000) considered to be responsible for the cell invasion shown by this bacterium. These same ORFs also exibit 94% similarity with ORFs carried by plasmids pCollB-P9 and pO157 of *E. coli* (Buchrieser et al., 2000). Another ORF (19) found to be present on plasmid E showed 82% similarity to ORF 52 which encodes an unnamed protein described in plasmid EAF (Tobe et al., 1999). The presence of plasmid EAF has been shown to greatly enhance the virulence of EPEC strains associated with diarrhoea in epidemiological studies (Donnenberg et al., 1992; Nataro and Kaper, 1998). An ORF (21) demonstrated 75% similarity to ORF 29 from the 50-kb virulence plasmid of *Salmonella enterica* (Haneda et al., 2001). These data suggest that plasmid E in a similar way to that described for pathogenicity-related plasmids also harbors pathogenicity-related sequences. These sequences may be responsible for the pathogenicity demonstrated by strains SEPT13 and TE. It is noteworthy that many of the virulence factors so far described for APEC strains were also related to the virulence of extraintestinal *E. coli* strains pathogenic to other host species (Rodriguez-Siek et al., 2005), suggesting that the basis for the virulence APEC strains may be similar to that of the strains described (Rodriguez-Siek et al., 2005). The cited authors demonstrated that APEC and UPEC strains share several virulence genes, suggesting that these strains may show similar adaptation for an extraintestinal lifestyle. This point of view would indicate that APEC strains could cause extraintestinal diseases in human beings.

ORFs possessing high similarity to genes related to the conjugative process were also found on plasmid E (Table 1). Among these, ORF 2 showed 100% similarity to ORF 4 which encodes the RepA helicase protein from plasmid RSF1010 of *E. coli* (Jezewska et al., 2004), and ORFs 8, 12 and 17, 18, and 22 demonstrated 97, 94, 90, 90, and 73%, respectively, similarity to genes *Tra*V/R, *fin*O, *Tra*X, *Tra*I, and *Tra*U (Manwaring et al., 1999).The similarities shown by these ORFs were expected because plasmid E is conjugative (Stehling et al., 2003a). Genetic horizontal transfer among *E. coli* and other Enterobacteriacea pathogenic strains has been found by *E. coli*-genome analysis. DNA sequencing studies showed that *E. coli* genome contains plasmid-related sequences, insertion sequence (IS) elements, phage remnants, and many other patches of unusual composition, indicating genome plasticity acquired through horizontal transfer (Burland et al., 1995; Blattner et al., 1997; Mokady et al., 2005; Wick et al., 2005; Herold et al., 2005). These mobile elements constitute an increasing health problem because they are associated with drug resistance and pathogenicity island horizontal transfer (Hacker and Kaper, 1999).

Plasmid E also exhibited ORFs with high similarity to chromosomal genes: similarity of 100% to ORF f276 from *E. coli* (ORF 1), 97% (ORFs 6 and 7) to a hypothetical ORF from *S. fexneri* and to ORF B from *E. coli*, and 93% (ORF 14) to a hypothetical ORF from *E. coli* O157:H7. Except for ORF 6 which encodes the IS150 insertion sequence (Haas and Rak, 2002), all the other ORFs encode hypothetical proteins (Burland et al., 1995; Perna et al., 2001).

This study showed that APEC strains harbor a large plasmid (43 MDa) with pathogenicity-related sequences that allow these strains to adhere to and invade avian tissues and to cause septicemic disease in poultry. The presence of sequence homology to other human pathogenic Enterobacteriaceae such as *E. coli* O157:H7, *Shigella* and *Salmonella* in conjugative plasmid E could indicate that horizontal genetic transfer occurs between strains from different host species, which could indicate that a certain degree of zoonotic risk would be present in APEC strains.

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