Epidemiol. Infect. (1996), **117**, 17–28

Distribution, gene sequence and expression *in vivo* of the plasmid encoded fimbrial antigen of *Salmonella* serotype Enteritidis

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(*Accepted 17 March 1996*)

SUMMARY

The *pef*A gene which encoded the serotype associated plasmid (SAP) mediated fimbrial major subunit antigen of *Salmonella enterica* serotype Typhimurium shared genetic identity with 128 of 706 salmonella isolates as demonstrated by dot (colony) hybridization. Seventy-seven of 113 isolates of Typhimurium and individual isolates of serotypes Bovis-morbificans, Cholerae-suis and Enteritidis phage type 9b hybridized *pef*A strongly, whereas 48 isolates of Enteritidis hybridized *pef*A weakly and one Enteritidis isolate of phage type 14b failed to hybridize. Individual isolates of 294 serotypes and 247 individual isolates of serotype Dublin did not hybridize *pef*A. Southern hybridization of plasmids extracted from Enteritidis demonstrated that the *pef*A gene probe hybridized strongly an atypical SAP of 80 kb in size harboured by one Enteritidis isolate of phage-type 9b, whereas the typical SAP of 58 kb in size harboured by 48 Enteritidis isolates hybridized weakly. One Enteritidis isolate of phage type 14b which failed to hybridize *pef*A in dot (colony) hybridization experiments was demonstrated to be plasmid free. A cosmid library of Enteritidis phage type 4 expressed in *Escherichia coli* K12 was screened by hybridization for the presence of *pef* sequences. Recombinant clones which were deduced to harbour the entire *pef* operon elaborated a PEF-like fimbrial structure at the cell surface. The PEF-like fimbrial antigen was purified from one cosmid clone and used in western blot experiments with sera from chickens infected with Enteritidis phage-type 4. Seroconversion to the fimbrial antigen was observed which indicated that the Enteritidis PEF-like fimbrial structure was expressed at some stage during infection. Nucleotide sequence analysis demonstrated that the *pef*A alleles of Typhimurium and Enteritidis phage-type 4 shared 76% DNA nucleotide and 82% deduced amino acid sequence identity.

INTRODUCTION

Takeuchi [1] described a two step process of association between salmonella with the gut epithelium prior to cellular (enterocyte) uptake and suggested that bacterial cell surface components, possibly fimbrial structures, may have initiated this association. Molecular approaches have identified a number of genetic loci involved in cellular association and uptake [2–4] and whilst these studies indicated that cellular association and uptake was multifactorial, evidence for a role for fimbrial surface structures in adherence and colonisation by salmonella has remained limited until recently.

Lockman and Curtiss [5] described a double mutant of Typhimurium deficient for the elaboration of flagella and type 1 fimbrial structures which exhibited a 50% lethal dose of 10^7 cfu in orally infected mice, whereas the wild type progenitor and single mutants deficient for the elaboration of either flagellae or type 1 fimbrial structures alone exhibited a 50% lethal dose of 2×10^4. The localized adherence (LA) pheno-

Table 1. *Distribution of* pef*A and* spv *gene sequences within* Salmonella enterica *serotypes Typhimurium, Enteritidis, Dublin, Cholerae-suis and Bovis-morbificans*

Phage Type	Number tested	Number *pef*A	Number *spv*$^+$	Phage Type	Number tested	Number *pef*A$^+$	Number *spv*$^+$
	Typhimurium				Typhimurium		
1	1	1	1	193	10	4	4
2	1	1	1	4	2	0	0
6	1	1	1	37	2	0	0
8	1	1	1	170	10	0	0
9	4	4	4		Enteritidis		
12	10	10	10	1	1	1	1
13	1	1	1	1a	1	1	1
18	1	1	1	4	26	26	26
41	1	1	1	4a	1	1	1
44	1	1	1	5	1	1	1
49	6	6	6	5a	1	1	1
49A	1	1	1	6	1	1	1
69	1	1	1	7	1	1	1
99	1	1	1	7a	1	1	1
103	1	1	1	8	4	4	4
104	4	4	4	9b	1	1	1
107	1	1	1	11	2	2	2
108	1	1	1	13	2	2	2
114	1	1	1	13a	1	1	1
141	1	1	1	14b	1	0	0
195	1	1	1	21	1	1	1
204	6	6	6	24	1	1	1
204A	1	1	1	29	1	1	1
204C	10	10	10	30	1	1	1
208	1	1	1	Atypical	1	1	1
10	6	3	3		Dublin		
40	6	2	2	Not typed	247	0	247
66	2	1	1		Cholerae-suis		
110	10	6	6	NA	1	1	1
146	2	1	1		Bovis-borbificans		
160	4	1	1	NA	1	1	1

The following serotypes were demonstrated by dot (colony) hybridization not to share genetic identity with *pef*A and are listed alphabetically by group [x]. [B] Abony, Abortus-equi, Abortus-ovis, Agama, Agona, Bradford, Brancaster, Brandenburg, Bredeney, Budapest, Chester, Coeln, Derby, Duisburg, Essen, Haifi, Hato, Helsinki, Heidelberg, Indiana, Java, Kalamu, Kiambu, Kimuenza, Massenya, Mons, Paratyphi-B, Reading, Saint-Paul, Salinatis, Sandiego, Schwartzengrund, Stanley, Stanleyville, Tyresoe, Wien; [C1/C4] Bareilly, Bonn, Braenderup, Concord, Colypark, Eimsbuettel, Georgia, Hartford, Infantis, Inganda, Isangi, Lille, Livingstone, Makiso, Mbandaka, Montevideo, Ness-ziona, Nigeria, Oakland, Ohio, Oranienberg, Oslo, Potsdam, Richmond, Singapore, Tennessee, Thompson, Virchow; [C2/C3] Aba, Alagbon, Albany, Altona, Belfast, Blockley, Bonariensis, Chomedy, Cleveland, Corvallis, Doncaster, Dusseldorf, Emek, Gold-coast, Haardt, Hadar, Hindmarsh, Istanbul, Kentucky, Kottbus, Litchfield, Manchester, Manhattan, Muenchen, Newport, Nagoya, Tado, Takoradi, Tallahassee, Tamale, Virginia; [D1] Berta, Blegdam, Canastel, Dar-es-salaam, Durban, Eastbourne, Gallinarum, Kapemba, Kim; [D2] Fresno, Inglis, Louisiana, Ontario, Plymouth, Strasbourg; [E1] Alfort, Amager, Anatum, Butantan, Falkensee, Give, Lamin, London, Lexington, Meleagridis, Muenster, Nchanga, Okefoko, Orion, Stockholm, Uganda, Vjele, Weltervreden, Westhampton, Yeerongpilly, Zanzibar; [E2] Binza, Dessau, Drypool, Goerlitz, Halmstad, Kinsasha, Lanka, Manila, New-Brunswick, New-haw, Newington, Portsmouth, Rosenthal, Tournai, Westminster; [E3] Arkansas, Canoga, Illinois, Minneapolis, Thomasville; [E4] Bedford, Broughton, Canstatt, Gnesta, Krefeld, Llandoff, Liverpool, Niloese, Seftenberg, Takasony, Visby; [F] Aberdeen, Chandans, Chingola, Senegal, Rubislaw, Tel-hashomer; [G1/G2] Ajiobo, Cubana, Durham, Grumpensis, Havana, Idikan, Jukestown, Kedougou, Mishmar-haemek, Okatie, Ordonez, Poona, Putten, Tel-el-kebir, Worthington, Wyldegreen; [H] Albuquerque, Caracus, Carrau, Florida, Fischerkietz; [I] Adeoyo, Brazil, Chameleon, Glasgow, Hannover, Hithergreen, Hull, Hvittingfoss, Kibi, Malstatt, Merseyside, Nottingham, Orientalis, Rowbarton, Salford, Tees; [J] Berlin, Bleadon, Jangwani, Kirkee; [K] Cerro, Memphis, Orlando, Pontypridd, Rawash, Toulon; [L] Ghana, Good, Minnesota, Ruiru, Soesterberg, Wandsbeck; [M] Ashanti, Brisbane, Chicago, Dakar, Dresden, Kibusi, Langford, Ona, Pomona, Selby, Tel-aviv, Vinohrady; [N] Godesberg,

type of enteropathogenic *Escherichia coli* was demonstrated to be mediated at least in part by the bundle forming pili encoded by the *bfp* gene cluster [6, 7]. Individual isolates of Typhimurium, Enteritidis and Dublin possessed the LA phenotype, hybridized a *bfp* gene probe derived from an EPEC isolate and, in the case of Dublin, elaborated BFP-like fimbrial structures on adherence to Hep-2 cell monolayers [7]. Contact of Typhimurium with cultured MDCK cells induced transient expression of an ill-defined surface appendage [8] and the long polar fimbrial (LPF) structure elaborated by Typhimurium [9] was shown to mediate adhesion of Typhimurium to Peyer's patches [10]. The 'virulence' plasmid of salmonella, more accurately described as serotype associated plasmid (SAP) [11] and this term will be used throughout this paper, has been shown to contribute to the systemic phase of Typhimurium infection of murines [12] and of Dublin infections of bovines [13]. The genetic loci, *spv*, contributing to this phenotype has been described [14]. However, Sizemore and others [15] generated a transposition mediated mutation of the SAP of Typhimurium in a region distinct from the *spv* virulence genes which resulted in reduced invasiveness, as determined by spleen counts, in the mouse model. The site of the mutation was located in or adjacent to a region which was demonstrated by Friedrich and coworkers [16] to encode determinants for the elaboration of the plasmid encoded fimbrial (PEF) structure. The PEF fimbrial structure has been demonstrated to mediate adhesion of Typhimurium to the murine small intestine by Baumler and others [17].

Our research has focused on the role of surface structures of Enteritidis in pathogenesis. Enteritidis has been demonstrated to harbour a SAP of 58 kb in size [18] and has been shown to elaborate at least four distinct fimbrial structures. The Type 1 fimbrial structure, also referred to as SEF21, has been demonstrated to bind laminin and promote mannose sensitive haemagglutination [19]. The SEF17 fimbrial structure, which was demonstrated to share 70% identity with the 'curli' fimbrial structure of *E. coli* [20], generated an aggregative phenotype and bound fibronectin [21–23]. The SEF14 fimbrial structure [24–27] was implicated in persistent infection in the chicken [28, 29]. The genetic determinants of the SEF'D' fimbrial structure, also referred to as SEF18, were colocated with the genes encoding SEF14 [30] but as yet no demonstrable phenotype has been ascribed to the SEF'D' fimbrial structure. Genetic evidence that Enteritidis isolates encode *lpf* and *bfp*-like sequences has been gained also [7, 9].

The SAPs have been demonstrated to share considerable genetic identity [31–34], have been shown to encode the *spv* region [18] and the SAP of Typhimurium was demonstrated to encode the PEF fimbrial structure [16]. We present evidence that the SAP of Enteritidis has the genetic potential for the elaboration of a PEF-like fimbrial structure, whereas the SAP of Dublin does not, and that the Enteritidis PEF-like structure elicits an immune response in infected chickens.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study comprised part of the international collection held in the Enterobacteriaceae section, Department of Bacteriology, Central Veterinary Laboratory. Confirmation of serotype [35] and phage type were as described previously [18] and are listed in Table 1. A field isolate of Enteritidis from an infected chicken, strain S1400/1994, which was characterized as phage type 4, was used to prepare a cosmid library. Strain DH5alpha (GIBCO-BRL, Life Technologies) was used as the standard *Escherichia coli* K12 cloning host. A field isolate of Typhimurium from an infected bovine, strain S1164/1994, which was characterized as DT 204c, was used to prepare the *pef* genes by PCR.

Media

Long term storage of salmonella isolates was on Dorset's egg slopes at room temperature. Luria-Bertani (LB) broth [36] or LB with agar added to a final concentration of 1·2% for solid medium was used for growth of all bacterial strains at 37 °C. Supplements were added as described [36]. Colonization factor agar (CFA, Difco) and buffered peptone water (pH 7·2) were used for growth of Enteritidis isolates and *E. coli* cosmid clone P4 prior to electron microscopical analysis.

Matopeni, Morehead, Urbana, Wayne; [O] Adelaide, Alachua, Ealing, Monschaui; [P] Foulpointe, Freetown, Inverness, Kidderminster, Perth; [Q] Champaign, Hofit, Mara, Wandsworth, Windemere; [R] Alsterdorf, Bulawayo, Johannesburg, Millesi, Omfisan, Ottershaw; [S] Ipswich, Offa, Waycross; [T] Freemantle, Gera, Toricada, Uphill, Waral; [U] Berkley, Milwaukee, Montreal; [V] Clovelly, Guinea, Niarembe, Vleuten; [W] Deversoir, Dugbe, Windhoek; [X] Bere, Bergen, Bilthoven, Bootle, Kaolack, Luke, Lyon, Quinhon; [Y] Dahlen, Djakarta; [Z] Flint, Greenside, Seaforth, Wassenaar.

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Plasmid analysis

Plasmid analysis was performed as described previously [18].

Genetic methods

Unless stated otherwise all genetic methods followed standard procedures as described by Sambrook and others [36]. Cloning vectors used were Bluescript KS$^+$ (Stratagene) for subcloning and pCRscript (Stratagene) for direct cloning of PCR products. Determination of DNA sequence was by the dideoxynucleotide chain termination reaction method (Sequenase™, Stratagene) and followed the manufacturer's recommendations.

Polymerase chain reaction (PCR) and cloning of amplicons

The polymerase chain method was performed as described by Saiki and others [37]. The primers used to amplify regions of the *pef* gene cluster were as follows:

PefA1
5′-TATAGGATCCGCCAATGAAGTAACTTTCCTGG-3′
PefA2
5′-TATAAGATCTTGTAAGCCACTGCGAAAGATGC-3′
PefB1
5′-TGAGCCGGAGATAATCCAA-3′
PefB2
5′-CTTATGAATAATAAACAACC-3′
PefI1
5′-AGAGCAATTCCGGTTTATGA-3′
PefI2
5′-TCAGAGCAGACGCCATAA-3′

and were designed based on the published sequence of Friedrich and others [16]. Primer pair PefA1/PefA2 was designed to amplify the entire *pef*A open reading frame which encoded the major surface antigen of the PEF fimbrial structure. Primer pairs PefB1/PefB2 and PefI1/PefI2 were designed to amplify putative regulatory genes, *pef*B and *pef*I, respectively. The conditions for PCR amplification were 40 cycles at 94 °C for 1·5 min, 60 °C for 1·5 min and 72 °C for 2·0 min in Saiki buffer adjusted to 5·5 mM Mg^{2+}. PCR products were cloned into pCRscript (Stratagene) and the procedures used were those recommended by the manufacturer. The template for the PCR reactions was purified DNA (10 ng) extracted from Typhimurium strain S1164/1994.

DNA-DNA hybridization

The methods for Southern transfer of plasmid profiles, dot (colony) hybridization and *spv* gene probe preparation were as described by Woodward and others [18]. *Eco*R I recognition sites flanked the PCR product cloning site of pCRscript. Cloned *pef*A, *pef*B and *pef*I amplicons were excised in separate experiments with *Eco*R I and the excised products were gel purified. Purified DNA was labelled to a high specific activity with ^{32}P dCTP (Megaprime, Amersham) and hybridization and post-hybridization washes carried out at high stringency as described previously [18].

Cosmid library preparation

SuperCos vector and a Gigapack XL II packaging kit (Stratagene) were used. SuperCos vector (6 μg) was digested for 16 h at 37 °C with 100 Units *Xba* I (Gibco, BRL). After digestion the restriction enzyme was removed by phenol/chloroform extraction and the vector DNA was precipitated by ethanol (70% volume) and sodium acetate pH 5·2 (300 mM). Self-ligation of the vector was prevented by removal of terminal phosphate groups by treatment with calf-intestinal phosphatase (Boehringer). The vector was digested again for 2 h at 37 °C with 10 Units of *Bam*H I (Gibco, BRL) per 1·2 μg DNA. The enzyme was removed and DNA precipitated as described above. Genomic DNA from Enteritidis S1400 was prepared using a rapid DNA extraction kit (Isoquick) and aliquots (1 μg) were digested for 1 h at 37 °C with doubling dilutions of *Sau*3A 1 (Gibco, BRL) in the concentration range of $1{\cdot}56 \times 10^{-2}$ to 10^{-4} Units. The restriction enzyme was removed by phenol/chloroform extraction, the genomic DNA was pooled and precipitated as described previously. The genomic DNA was dissolved in sucrose gradient buffer (10 mM TRIS pH 8·0, 10 mM sodium chloride, 1 mM EDTA) and loaded onto sucrose gradient (10–40%). Centrifugation was at 22000 g for 16 h at 20 °C (Beckman Ultracentrifuge, Ti40 swing out rotor). Fractions (150–200 μl) were collected and samples of each fraction were subjected to agarose gel electrophoresis. Samples containing genomic DNA fractionated in the size range 30–45 kb were diluted 1/3 in 10 mM TRIS pH 8·0, 1 mM EDTA and precipitated as described above. Calf intestinal phosphatase was used as above to prevent self-ligation of the genomic DNA. Ligation of SuperCos DNA (1 μg) and genomic DNA (2·5 μg) in a 20 μl reaction volume containing buffer (0·5 M

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TRIS-HCl pH 9·3, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermine) and 3 Units of T4 DNA Ligase (Promega) was at 14 °C for 16 h. Ligated DNA was packaged (Gigapack XL II packaging kit, Stratagene) and the cosmid library was titered in accordance with the manufacturer's recommendations. The library was amplified and 480 single colonies were picked and resuspended individually in 180 μl LB + Kanamycin (50 μg ml^{-1}) and grown for 16 h at 37 °C. Samples (65 μl) were removed and placed into the wells of microtitre plates to which fresh LB + Kanamycin (110 μl) had been added. The plates were incubated at 37 °C for 5 h after which glycerol was added to a final concentration of 15% v/v. The plates were stored frozen at −70 °C. To prepare the cosmid library for colony hybridization, the microtitre plates were thawed and samples of each recombinant were inoculated by a replica plating apparatus (Whiteley) onto Hybond-N nylon membrane (Amersham) overlaid on LB + Kanamycin (50 μg ml^{-1}) agar followed by incubation at 37 °C for 16 h. Dot (colony) lysis and hybridization were as described above.

Electron microscopy

Bacteria were streaked onto CFA and inoculated into in buffered peptone water (pH 7·2) supplemented with the appropriate antibiotic and grown overnight at 37 °C. The bacterial cells were harvested, washed with 1 × PBS, allowed to adhere to Formavar-carbon coated grids (CVL, Addlestone) for 10 min and negatively stained with 2% phosphotungstic acid (pH 6·6) for 15 s.

Purification of PEF fimbrial antigen

Escherichia coli cosmid clone P4 was confirmed *pef*A$^+$ *pef*B$^+$ *pef*I$^+$ by hybridization and confirmed by electron microscopy to express PEF-like fimbrial structures and was grown overnight in buffered peptone water (pH 7·2) supplemented with the appropriate antibiotic at 37 °C. Bacterial cells were harvested by centrifugation and resuspended in PBS/TRIS. Fimbrial structures were detached from the cells by the method of Thorns and other [27] and the cell debris was removed by centrifugation at 13000 g for 30 s in a microfuge (MSE). The supernatant was passed through a Diaflow Ultrafiltration membrane YM10 filter (Amicon Inc.) to produce a concentrated crude preparation of fimbrial antigen. The crude preparation was fractionated by SDS–PAGE (15% Acrylamide) and a test strip of the gel was stained with Coomassie Blue to identify the protein product of interest. A gel strip containing the protein product of interest was excised and electro-eluted according to the method of Leppard and others [38]. Gel fractionation and electroelution was repeated twice to give a purified PEF sample.

Western blot analysis of PEF

The methods of Cooper and others [39] were used. Five-day-old specific pathogen free chicks were dosed orally with 10^5 cfu Enteritidis strain LA5 [39] and sera was taken at 8 weeks of age post-inoculation. Purified PEF antigen and SEF14 antigen purified as described previously [27] were fractionated by SDS–PAGE (12·5% Acrylamide) and the separated antigens were transferred to a nitocellulose membrane (Sartorius) at 300 mA for 1 h in a CAPS-methanol transfer buffer. The membrane was air dried, cut into strips, blocked with TBST (TRIS-HCl 100 mM, NaCl 0.9% w/v, Tween-20 0·1% w/v) supplemented with 3% Marvel™ and washed with TBST. Sera diluted 1/50 in TBST supplemented with 3% Marvel™ was used as the primary antibody probe for 1 h at 37 °C. Development was with alkaline phosphatase labelled anti-chicken IgG (Amersham) and followed manufacturer's recommendations.

RESULTS

Distribution of *pef*A sequences

To determine the distribution of *pef* like sequences within the genus *Salmonella*, a gene probe specific for *pef*A, previously demonstrated to encode the major fimbrial subunit protein of the PEF fimbrial structure [16], was prepared by the polymerase chain reaction (PCR). DNA extracted from Typhimurium DT204c strain S1164/1994 was used as template and a PCR product of the predicted size, 460 bp, was identified by agarose gel electrophoresis. The PCR product was cloned into pCRscript (Stratagene) and the DNA sequence determined to be identical to the published sequence [16]. The *pef*A gene was excised from pCRscript and labelled to high specific activity with ^{32}P dCTP and used in dot (colony) hybridization experiments with a total of 299 *Salmonella* serotypes (706 isolates). Of 133 Typhimurium isolates, 77 hybridized *pef*A (Table 1) as did individual isolates of Bovis-morbificans, Cholerae-suis and Enteritidis

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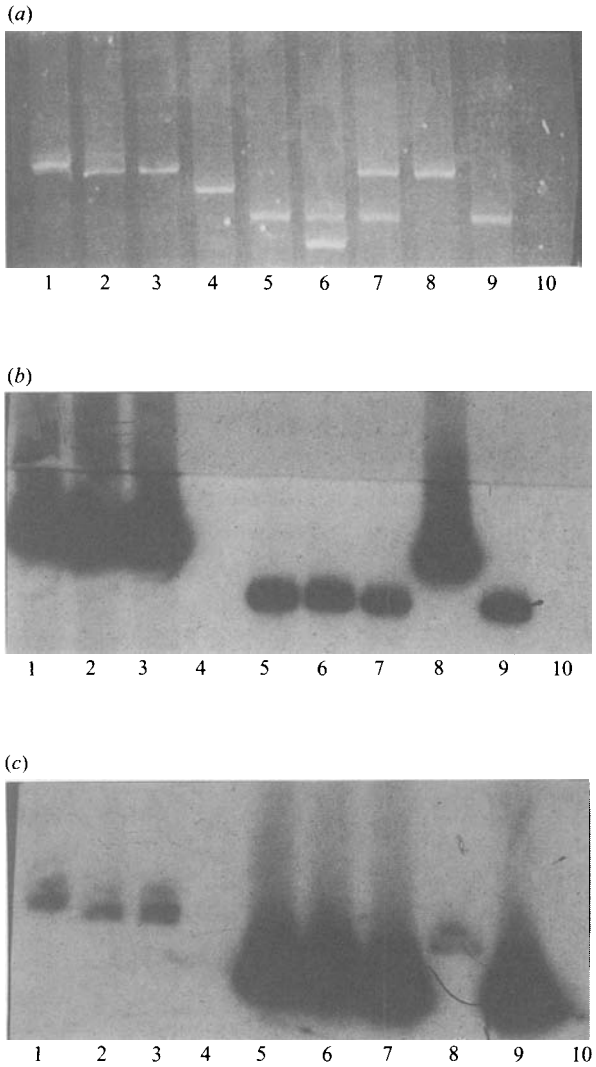

Fig. 1. Southern hybridization of plasmid profiles of Typhimurium and Enteritidis isolates with two distinct *pef*A alleles as gene probes. (*a*) Plasmid profiles from isolates Typhimurium DT204c isolate 1721 (lane 1), Typhimurium DT 49 isolate 3292 (lane 2) Typhimurium DT110 isolate 1191 (lane 3), Dublin isolate S368/94 (lane 4), Enteritidis phage type 13 isolate S10-13 (lane 5), Enteritidis phage type 24 isolate S10-24 (lane 6), Enteritidis phage type 4 isolate S10-1485 (lane 7), Enteritidis phage type 9b isolate S10-9b (lane 8), Enteritidis phage type 1 isolate S10-1 (lane 9) and Enteritidis phage type 14b isolate S10-14b. Isolate designation refers to the CVL type culture collection number. The plasmid profiles were hybridized in separate experiments with the *pef*A allele derived from Typhimurium (*b*) and the *pef*A allele derived from Enteritidis (*c*). The autoradiographs were overexposed to show the relative intensity of hybridization between alleles.

phage type 9b. Of a further 49 Enteritidis isolates, 48 hybridized weakly whereas the single isolate of phage type 14b tested in this work failed to hybridize (Table 1). Individual isolates of 293 other *Salmonella* serotypes and 247 isolates of Dublin did not hybridize *pef*A.

Association between *pef*A and *spv*

To assess the association between *pef*A and *spv*, colony hybridization experiments with Typhimurium, Enteritidis, Bovis-morbificans, Cholerae-suis and Dublin isolates and the *spv* probe were done. Without exception, all *pef*A$^+$ isolates of Typhimurium, Enteritidis, Bovis-morbificans and Cholerae-suis hybridized *spv* (Table 1), whereas all 247 Dublin isolates hybridized *spv* but not *pef*A.

The *pef*A allele of Enteritidis was encoded by the serotype associated plasmid

To test that the *pef* region in Enteritidis isolates was encoded by the SAP, Southern transfers of plasmid profiles from representative phage types of both Typhimurium and Enteritidis (Fig. 1*a*) were hybridized with the *pef*A gene probe. Plasmids of 90 kb in size harboured by the Typhimurium isolates (Fig. 1*b*, lanes 1, 2, 3) and a 90 kb plasmid harboured by Enteritidis phage type 9b (Fig. 1*b*, lane 8) hybridized strongly. Plasmids of 58 kb in size harboured by the Enteritidis isolates (Fig. 1*b*, lanes 5, 6, 7, 9) hybridized weakly. The Enteritidis phage type 24 isolate (Fig. 1, lane 6) harboured an additional plasmid of 36 kb in size and the Enteritidis phage type 4 isolate (Fig. 1, lane 7) harboured an additional plasmid of 90 kb in size. Neither of these additional plasmids hybridized the *pef*A gene probe nor the *spv* probe (see below). No further analysis of these additional plasmids was undertaken. The Enteritidis phage type 14b isolate (Fig. 1, lane 10) was demonstrated to be plasmid free and the 72 kb SAP derived from Dublin isolate S9/1994 (Fig. 1, lane 4) did not hybridize *pef*A as anticipated.

Nucleotide sequence analysis of the Enteritidis *pef*A allele

The hybridization data indicated that the 58 kb SAP of Enteritidis encoded a sequence which shared incomplete identity with the Typhimurium *pef*A gene, possibly a *pef*A allele. Thus, the PCR described above was used to amplify the putative *pef*A allele from Enteritidis S1400/1994. The conditions for the PCR were as described in methods and the template was purified total genomic DNA (10 ng) extracted from

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                    v10        v20        v30        v40        v50
PEFAENT.SEQ ATGAAAAAGAGCATTATTGCTTCCATTATTGCACTGGGTGTTCTGGGCGG
            ATGAAAAAGAGCATTATTGCTTCCATTATTGCACTGGGTGTTCTGGGCGG
PEFASTM.SEQ ATGAAAAAGAGCATTATTGCTTCCATTATTGCACTGGGTGTTCTGGGCGG
                    ^10        ^20        ^30        ^40        ^50

                    v60        v70        v80        v90        v100
PEFAENT.SEQ GACTGCACACGCTGCCAATGAAGTGACCTTCCTGGGTTCAGTGAGCGCCA
            GACTGCACACGCTGCCAATGAAGT AC TTCCTGGGTTCAGT AGCGC A
PEFASTM.SEQ GACTGCACACGCTGCCAATGAAGTAACTTTCCTGGGTTCAGTAAGCGCAA
                    ^60        ^70        ^80        ^90        ^100

                    v110       v120       v130       v140       v150
PEFAENT.SEQ CGACCTGTGACCTGACCACTTCTGTCAACGGCGCAGCTGGGCCAAACCAG
            C ACCTGTGACCTGACCACTTCTGTCAACGGCGG GCT  GCC AACCAG
PEFASTM.SEQ CAACCTGTGACCTGACCACTTCTGTCAACGGCGCCGCTCAGCCGAACCAG
                    ^110       ^120       ^130       ^140       ^150

                    v160       v170       v180       v190       v200
PEFAENT.SEQ GTTGTTCAGTTAGGTACTGTTCAGGCAGGTCAGGAAGGTACGGCTGTTGC
            GTTGTTCAG T GGTAC GTTCAGGC    CAG   GGTA    TGTTG
PEFASTM.SEQ GTTGTTCAGCTGGGTACCGTTCAGGCTAACCAGCCGGGTAATTTTGTTGA
                    ^160       ^170       ^180       ^190       ^200

                    v210       v220       v230       v240       v250
PEFAENT.SEQ TTTTGCCATCAAGCCGGCTAACCCGGGCTCCCTGGGGTGTCAGGGGCTGG
             TT GCCAT AA CCGG T ACCCG    C C GGG TGT      CTGG
PEFASTM.SEQ CTTCGCCATGAAACCGGTTGACCCGAATGCACAGGGTTGTGCAAATCTGG
                    ^210       ^220       ^230       ^240       ^250

                    v260       v270       v280       v290       v300
PEFAENT.SEQ ATACTAAAACCGTCACTGTCTCCTGGGCTTCTGCTGCCCTGAATGCTGAC
             T   AAAAC G CACTGT TCCTGGGCTTCTGCTGCCCTG ATG  GA
PEFASTM.SEQ CTCAGAAAACGGCCACTGTTTCCTGGGCTTCTGCTGCCCTGGATGGCGAA
                    ^260       ^270       ^280       ^290       ^300

                    v310       v320       v330       v340       v350
PEFAENT.SEQ GGCTTTGGTGCAACTGGCGGCGCAGCAACAGACGCTACTGTTCTGGTCAA
            GGCTTTGG GCAAC  GCGG  CAGC AC GA GC A  GT CTGGT  A
PEFASTM.SEQ GGCTTTGGGGCAACCAGCGGTACAGCTACTGATGCCAAAGTACTGGTTGA
                    ^310       ^320       ^330       ^340       ^350

                    v360       v370       v380           v390
PEFAENT.SEQ TAACGTGAACGCAAAAACCAATCCTGGTGCGG----CGGTGAATGCAAAT
             A CGTGAAC C AAAA C   CC GG GC G    CG T AA G
PEFASTM.SEQ AAGCGTGAACTCCAAAAAC---CCGGGGGCTGTAAACGCT-AACG-----
                    ^360           ^370       ^380       ^390
                 v400       v410       v420       v430       v440
PEFAENT.SEQ GCCAGCACCGTGGAATTTAACGGTGCTGATCTGAACACCGACGGCCTGAA
             CCAGCAC GT GA TTT A GGTGC  A CTGA CACCGACGGCCTG A
PEFASTM.SEQ -CCAGCACGGTTGACTTTGAAGGTGCCAAGCTGACCACCGACGGCCTGCA
                    ^400       ^410       ^420       ^430       ^440

                 v450       v460       v470       v480       v490
PEFAENT.SEQ ATTCCAGGCCAAACTGAAAGGTGGCCAGACTGAAGGTGACTTCAAATCTG
             TTC A GCCAA CTGAAAGGTGG   GAC GAAGGTGACTTCAA TCTG
PEFASTM.SEQ GTTCAAAGCCAAGCTGAAAGGTGGTGCGACCGAAGGTGACTTCAAGTCTG
                    ^450       ^460       ^470       ^480       ^490

                 v500       v510       v520
PEFAENT.SEQ TGGCATCTTTCGCAGTGGCTTACAAATAA
            TGGCATCTTTCGCAGTGGCTTACAAATAA
PEFASTM.SEQ TGGCATCTTTCGCAGTGGCTTACAAATAA
                    ^500       ^510
```

Fig. 2. Nucleotide sequence alignment of the open reading frames of the Enteritidis (upper) and Typhimurium (lower) *pef*A alleles. Accession number X97448.

Enteritidis S1400/1994. A PCR product of the predicted size for a *pef*A allele was generated and cloned.

Preliminary sequence analysis of the Enteritidis *pef*A allele derived by PCR indicated *c.* 80% sequence identity with the Typhimurium *pef*A allele. However, because PCR may have introduced error, the sequence was confirmed by analysis of the native *pef*A allele derived from an Enteritidis phage type 4 cosmid library prepared as described in Methods. Of 480

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```
                10v        20v        30v        40v        50v
PEFAENT   MKKSIIASIIALGVLGGTAHAANEVTFLGSVSATTCDLTTSVNGAAGPNQV
          MKKSIIASIIALGVLGGTAHAANEVTFLGSVSATTCDLTTSVNGAA PNQV
PEFASTM   MKKSIIASIIALGVLGGTAHAANEVTFLGSVSATTCDLTTSVNGAAQPNQV
                10^        20^        30^        40^        50^
               60v        70v        80v        90v       100v
PEFAENT   VQLGTVQAGQEGTAVAFAIKPANPGSLGCQGLDTKTVTVSWASAALNADGF
          VQLGTVQA.Q G. V.FA:KP.:P.: GC..L. KT.TVSWASAAL:::GF
PEFASTM   VQLGTVQANQPGNFVDFAMKPVDPNAQGCANLAQKTATVSWASAALDGEGF
               60^        70^        80^        90^       100^
             110v       120v       130v       140v       150v
PEFAENT   GATGGAATDATVLVNNVNAKTNPGAAVNANASTVEFNGADLNTDGLKFQAK
          GAT:G:ATDA.VLV::VN:K NPGA VNANASTV:F:GA.L.TDGL:F:AK
PEFASTM   GATSGTATDAKVLVESVNSK-NPGA-VNANASTVDFEGAKLTTDGLQFKAK
             110^       120^       130^       140^       150^
            160v       170v
PEFAENT   LKGGQTEGDFKSVASFAVAYKX
          LKGG.TEGDFKSVASFAVAYKX
PEFASTM   LKGGATEGDFKSVASFAVAYKX
               160^       170^
```

Fig. 3. Deduced amino acid sequence alignment of the Enteritidis (upper) and Typhimurium (lower) *pef*A antigens.

cosmid clones probed with the Enteritidis *pef*A allele, 32 recombinants hybridized which indicated a biased library with over-representation of SAP DNA sequences. The cosmid DNA from one clone, designated P4, was extracted and a 3·5 kb *Eco*R I/*Sal* I fragment which hybridized the *pef*A allele was subcloned into Bluescript KS$^+$. The nucleotide sequence of the *pef*A allele open reading frame was determined (Fig. 2) and compared with the published *pef*A sequence of Typhimurium [16]. The two alleles shared 76% DNA sequence homology with near perfect identity at the 3′ and 5′ termini, hence the success of the PCR. The Enteritidis *pef*A allele was determined to be six nucleotides longer than that of Typhimurium with significant sequence divergence located in three regions, base pairs 170–200, 220–250 and 360–400. The deduced amino acid sequences of the two alleles (Fig. 3) shared 82% homology which suggested that there might be significant antigenic differences between the PEF antigens of Typhimurium and Enteritidis.

The Enteritidis *pef*A allele was used in a reciprocal hybridization experiment in which Southern transfers of plasmid profiles prepared above (Fig. 1*a*, *c*). The Enteritidis *pef*A allele hybridized strongly the 58 kb SAP harboured by Enteritidis and weakly with the 90 kb SAPS of Typhimurium and Enteritidis phage type 9b.

The *pef* region of Enteritidis directed the synthesis of fimbriae

Of 50 isolates of Enteritidis, 49 were demonstrated by colony hybridization to possess a *pef*A allele encoded on the SAP and the question arose as to whether these Enteritidis isolates elaborated PEF-like fimbrial structures. It was reasoned that the entire *pef* gene cluster was required to enable synthesis of the fimbrial structure. Thus, hybridization experiments were undertaken to establish the presence of other *pef* genes. Gene probes specific for *pef*B and *pef*I genes which flanked the *pef* gene cluster [16] were prepared by PCR as described in Methods and used as gene probes in colony hybridization experiments with Enteritidis isolates and cosmid clones encoding *pef*A. All 49 Enteritidis which encoded *pef*A hybridized both *pef*B and *pef*I as did cosmid clone P4 which indicated the presence of the entire *pef* gene cluster.

Electron microscopical analysis of cosmid clone P4 (Fig. 4) demonstrated the elaboration of a fimbrial structure which was absent from controls which were the *Escherichia coli* cloning host and the cloning host harbouring the cosmid vector without insert. Similar fimbrial structures were not visualized by electron microscopical analysis of any of six Enteritidis isolates which had been demonstrated to encode the *pef* gene region by colony hybridization. Experiments to establish conditions for *in vitro* expression of PEF by Enteritidis were not undertaken.

The Enteritidis *Pef* was expressed *in vivo* in the chicken

Preliminary electron microscopical analysis did not demonstrate elaboration of the PEF fimbrial structure by Enteritidis under the *in vitro* conditions used. Whether the PEF fimbrial structure of Enteritidis was expressed *in vivo* during infection of chickens was tested by western blot experiments. One-day-old chicks, eight in total, were dosed orally with Enteritidis strain LA5 and sera was prepared 56 days post

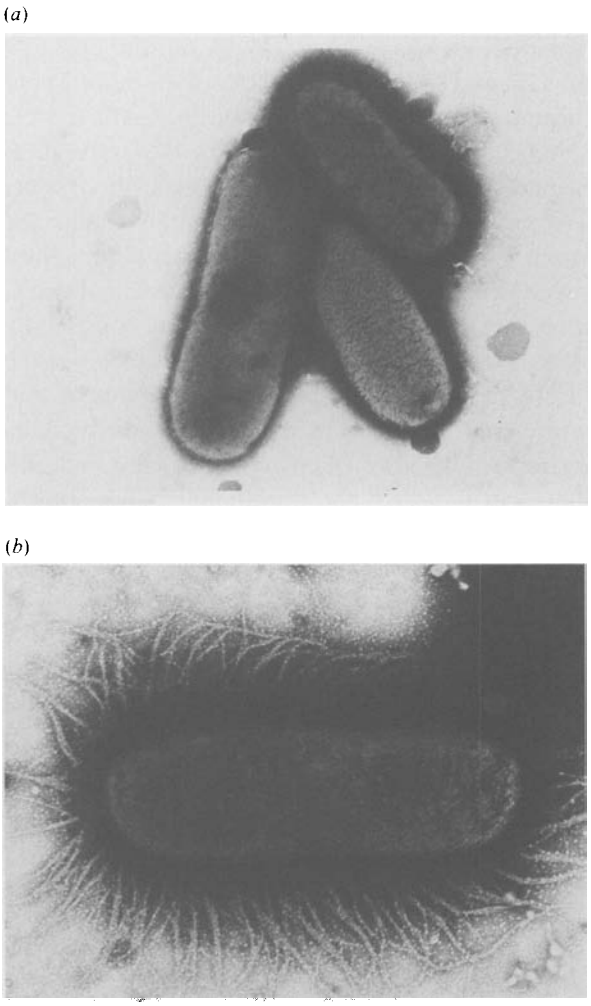

Fig. 4. Electron micrographs showing representative bacterial cells of *Escherichia coli* K12 strain DH5 alpha (Life Technologies) either without (*a*) or with (*b*) the *pef* gene cluster derived from Enteritidis phage type 4 strain S1400. Magnification × 36000.

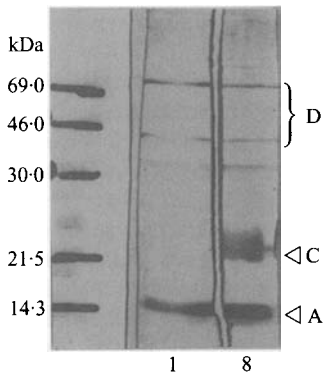

Fig. 5. Western blots using sera from chicks at 30 days post infection with Enteritidis phage type 4 isolate LA5 to probe semi-purified SEF14 and PEF antigens. Track one shows molecular size markers, tracks two and three show the reaction of sera from chicks 1 and 8, respectively, to SEF14 (A) and PEF monomeric (C) and multimeric (D) forms.

inoculation from each of the eight birds. Strain LA5 was shown by hybridization to encode the *pef* gene region and was used in chicken infection studies routinely in this laboratory [39]. The individual sera were blotted against the PEF antigen (Fig. 5). The internal control included in these experiments was the SEF14 fimbrial antigen against which a B-cell response is mounted by the chicken [25, 27]. Five sera probed both PEF and SEF14 (Fig. 5, lane 8), whereas three sera probed SEF14 but not PEF (Fig. 5, lane 1).

DISCUSSION

This work demonstrated that the typical 58 kb SAP of Enteritidis encoded a *pef*A allele which shared *c.* 80% genetic identity with the *pef*A allele of Typhimurium. Four lines of evidence indicated that the typical 58 kb SAP of Enteritidis encoded the entire *pef* gene cluster. Firstly, the SAPs of Typhimurium and Enteritidis have been demonstrated to share considerable genetic identity in this region [33]. Secondly, the data presented in this paper demonstrated hybridization of Enteritidis isolates with three different genes associated with this cluster, namely *pef*A, *pef*B and *pef*I. Thirdly, SAPs from Enteritidis isolates hybridized both *pef*A and *spv*. Fourthly, cosmid clones encoding Enteritidis derived *pef* regions elaborated fimbrial structures. However, it should be borne in mind that these fimbriae may have been cryptic *E. coli* fimbriae induced upon the introduction of the cosmid and not PEF. A similar phenomenon was observed by Arnquist and others [40] in the genetic analysis of the 'curli' fimbrial structure of *E. coli*.

PEF-like fimbrial structures were elaborated *in vitro* by *E. coli* harbouring the *pef* region as part of a cosmid whereas similar fimbrial structures were not elaborated by Enteritidis grown under the same *in vitro* conditions. This suggested that genes distant from the *pef* gene cluster, possibly located on the bacterial chromosome, may regulate expression of the PEF fimbrial structure by Enteritidis. Chromosomal involvement for the expression of *spv* genes has been demonstrated [41, 42]. Additionally, Friedrich and others [16] commented on the unusual gene arrangement within the *pef* gene cluster with the putative regulatory function encoded by *pef*I being located downstream and transcribed in the same direction as the other genes.

An immune response to the PEF fimbrial antigen was elicited in the chicken albeit not as completely as with another fimbrial antigen, SEF14, which did elicit

https://doi.org/10.1017/S0950268800001084 Published online by Cambridge University Press

a strong immune response in the same host. However, this evidence for *in vivo* expression of the PEF fimbrial antigen by Enteritidis and, combined with the apparent lack of *in vitro* expression, indicated that a regulatory mechanism linked with *in vivo* stress may have operated. If mature fimbriae were expressed *in vivo*, then these structures may have played a role in the pathogenesis of Enteritidis in the chicken. Sizemore and others [15] described transposon Tn5 insertional mutations of the Typhimurium SAP adjacent to, if not in, the *pef* region which were reduced for virulence in the mouse model and Baumler and others [17] generated *pef*C mutants of Typhimurium which adhered to the murine small intestinal epithelium with reduced efficiency compared with the isogenic wild type. Whereas these data indicated the Typhimurium PEF was a virulence determinant in the mouse, a role for the Enteritidis PEF fimbrial structure in colonization of the chicken remains to be elucidated.

Detailed physical analyses of the SAPs have demonstrated considerable genetic identity [31–34]. Therefore, it was not unexpected that *pef*A alleles were encoded by Bovis-morbificans, Cholerae-suis, Enteritidis and Typhimurium which have been demonstrated previously to harbour SAPs [18]. The absence of *pef* from Dublin isolates, however, indicated a distinct evolutionary pathway for the 72 kb SAP of Dublin. Of the *pef*A alleles encoded by Enteritidis, only that of phage type 9b shared a high degree of identity with the *pef*A alleles of Cholerae-suis, Bovismorbificans and Typhimurium, and this allele was encoded by an atypical SAP of 80 kb in size. Rankin and others [43] described an 80 kb SAP, pOG690, from an Enteritidis phage type 9b isolate and suggested that this plasmid may be a direct evolutionary intermediate in the descent of virulence plasmids from Typhimurium to Enteritidis. The data presented here support this hypothesis. Another facet of the evolution of the SAPs is the physical colocation of the *spv* virulence determinants with *pef* fimbrial determinants which prompted the question as to whether these gene clusters function in pathogenesis in concert.

We have reported previously [18] the distribution of the *spv* region within Typhimurium phage types. The hybridization data presented here indicated that the colocation of both *spv* and *pef*A in this serotype, at least, is absolute. Additionally, these data have confirmed our previous findings [18] that that all isolates of Typhimurium phage types 4, 37 and 170, were SAP free, whilst about 50% of isolates of Typhimurium phage types 10, 40, 66, 110, 146, 160 and 193, and approaching 100% of all other Typhimurium isolates tested, harboured the SAP.

Nucleotide sequence analysis of the *pef*A alleles revealed significant differences, the majority of which were associated with changed deduced amino acid sequence rather than neutral genetic drift at the third, or wobble, base of the codon. The N-terminal and C-terminal regions of the *pef*A alleles were conserved, a feature noted of *fli*C alleles also [44], which possibly reflected selective pressure on those regions to fold, mature and present the molecule to the building apparatus. Whether the extent of the sequence differences indicated the evolutionary divergence as being very ancient is open to considerable debate.

The function of PEF, as well as the many other fimbriae, of Enteritidis remains unclear. However, the closely related PEF fimbrial structure of Typhimurium has been demonstrated to mediate adhesion to the murine small intestinal epithelium [17] which may be indicative of a similar role of the Enteritidis PEF fimbrial structure. Direct evidence for their role in the infectious process of Enteritidis remains to be determined.

ACKNOWLEDGEMENT

The authors acknowledge support through the OZ01 program of the Ministry of Agriculture, Fisheries and Food, UK.

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