Detection of virulence determinants in clinical strains of *Salmonella enterica* serovar Enteritidis and mapping on macrorestriction profiles

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A total of 80 strains of *Salmonella enterica* serovar Enteritidis, causing gastroenteritis (G) or bacteraemia (B), and three control strains (C), were subjected to: (i) detection of 14 chromosomally and 1 plasmid-located virulence genes by PCR, (ii) detection of DNA polymorphisms by *Xba*I and *Bln*I PFGE, and cluster analysis, (iii) mapping of the 15 screened sequences on macrorestriction profiles and (iv) comparison of the screening and mapping results with data available for other *Salmonella* strains. Identical virulence genotypes and very similar macrorestriction profiles were shown by most *S. Enteritidis* strains. However, a number of B strains belonged to genomic types with polymorphisms affecting fragments carrying (SPI2-*slyA*), (SPI2-*slyA-phoP/Q-agfA*), (SPI4 and/or *stn*) and *spvC*. The information obtained provides the basis for further studies on the genetic background of virulence and the molecular epidemiology of *S. Enteritidis*.

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INTRODUCTION

Nontyphoidal *Salmonella* serovars are one of the leading causes of food-transmitted infections in developed countries. In most cases, infection results in gastroenteritis, usually a self-limiting illness. However, bacteria can also spread beyond the intestine, and cause systemic infections involving the bloodstream and/or various body organs. To cause disease, *Salmonella* relies on multiple virulence factors, many of which are clustered within *Salmonella* pathogenicity islands (SPIs) (Marcus *et al.*, 2000; Fierer & Guiney, 2001; Hensel, 2004). SPI1 and SPI2 each encode a structurally and functionally different type III secretion system, which is responsible for the delivery of effector proteins into the host cell cytosol. SPI3 is necessary for survival within macrophages and growth in low Mg²⁺ environments during the systemic phase of the disease. SPI4 is suspected to be required for intramacrophage survival and may also contribute to toxin secretion. Like SPI1, SPI5 appears mainly associated with enteropathogenesis, being involved in inflammation and chloride secretion. Some of the genes not residing in SPIs that also play a role in disease are *slyA*, *phoP/Q*, *agfA* and *stn*, encoding a transcriptional regulator of virulence genes required for survival within macrophages (initially identified as salmolyisin) (Watson *et al.*, 1999), a two-component global transcriptional regulator of *Salmonella* virulence (Miller, 1991), the thin aggregative fimbriae

(Collinson *et al.*, 1996a, b) and an enterotoxin (Prager *et al.*, 1995), respectively. Furthermore, many *Salmonella* serovars harbour virulence (V) plasmids with variable size, depending on the serovar (Rotger & Casadesús, 1999; Marcus *et al.*, 2000; Fierer & Guiney, 2001). All V plasmids share a highly conserved 8 kb region with five genes designated *spvRABCD* (*Salmonella* plasmid virulence). The *spv* region appears to promote rapid growth and survival of *Salmonella* within the host cells, being important for systemic infection in experimental animals.

During the last two decades, *Salmonella enterica* serovar Enteritidis has surpassed *Salmonella enterica* serovar Typhimurium as the most common serovar reported in European countries, including Spain (<http://www.eurosurveillance.org>). In addition to gastroenteritis, the incidence of bacteraemia caused by this serovar has also increased and, nowadays, it is far more often associated with invasive infections than serovar Typhimurium (Threlfall *et al.*, 1992; Le Bacq *et al.*, 1994; Rodríguez *et al.*, 1998; Ispahani & Slack, 2000). In the present work, *S. Enteritidis* strains representing the most frequent or most distinct types (based on random amplified polymorphic DNA typing, antimicrobial resistance, and plasmid and integron profiles), which have been causing gastroenteritis or bacteraemia in Asturias (Spain), were further characterized by virulence-gene profiling, focusing on virulence determinants that have been shown to be relevant for *S. Typhimurium* pathogenesis, and by macrorestriction-PFGE analysis, using two different

Abbreviation: SPI, *Salmonella* pathogenicity island.

endonucleases (*Xba*I and *Bln*I). The detected V genes were then mapped on the macrorestriction profiles, and their location compared with data available for other *Salmonella* strains (Collinson *et al.*, 1996b; McClelland *et al.*, 2001).

METHODS

Bacterial strains. A total of 3 control strains and 80 clinical isolates of *S. Enteritidis* were used in this study (Table 1). Each clinical isolate could be considered as a strain since they had been recovered from different patients, at different times, and no epidemiological relationship had been recorded among patients. A total of 40 strains were recovered from faeces of patients with gastroenteric episodes (G group), and another 40 from the blood of bacteraemia patients (B group). They represented the most frequent or distinct types

involved in human salmonellosis, recorded in Asturias, Spain, over the last decade (Landeras & Mendoza, 1998; Rodríguez *et al.*, 1998; Soto *et al.*, 2003; unpublished data).

PCR-based procedures. Aliquots of Luria–Bertani (LB) broth overnight cultures of the strains were the source of template DNA. Primers for SPIs were designed by computerized analysis of the sequences recorded under the accession numbers or references listed in Table 2, following criteria such as gene specificity, number of base pairs, annealing temperature and size of the amplification product. Primers for other screened genes have been reported previously (Doran *et al.*, 1993; Guerra *et al.*, 2000). In total, 15 primer pairs (Table 2) were used either alone or in the following combinations (for multiplex PCR): set I (*orgA*, *ssaQ*, *misL*, *spi4D* and *pipA*), set II (*invE/A*, *trrC*, *mgtC*, *spi4R* and *agfA*) and set III (*sopB*, *slyA*, *phoP/Q*, *stn* and *spvC*). Assays were performed in 50 µl reaction mixtures containing 2 µl template DNA, 200 µM each dNTP (Roche

Table 1. Features of the *Salmonella* serovar Enteritidis strains used in this work

Strain	<i>Xba</i> I profile	Phage type*	Plasmid* (kb/gene)	Representative strain† (no. of strains)
Faeces strains (<i>n</i> =40)	X2	PT1	60/ <i>spv</i>	LSP 27/00 (5)
	X2	PT4	60/ <i>spv</i>	LSP 280/00 (5)
	X2	PT4	60/ <i>spv</i> , 90/ <i>tem1</i>	LSP 34/00 (1)
	X2	PT6	60/ <i>spv</i>	LSP 380/00 (1)
	X2	NT	60/ <i>spv</i>	LSP 139/00 (3)
	X2	NT	60/ <i>spv</i> , 19/–, 7/ <i>tem1</i>	LSP 87/00 (1)
	X3	PT6a	60/ <i>spv</i> , 40/ <i>tem1</i>	LSP 54/00 (12)
	X3	PT6a	60/ <i>spv</i> , 90/ <i>tem1</i> , 40/–	LSP 39/00 (1)
	X3	PT6a	60/ <i>spv</i> , 90/–, 40/ <i>tem1</i>	LSP 201/00 (3)
	X3	PT6a	60/ <i>spv</i> , 100/ <i>tem1</i> , 40/ <i>tem1</i>	LSP 49/00 (1)
	X4	PT4	60/ <i>spv</i> , 90/ <i>tem1</i>	LSP 98/00 (2)
	X4	PT1	60/ <i>spv</i> , 90/ <i>tem1</i> , 5/–	LSP 381/00 (1)
	X4	PT6	60/ <i>spv</i> , 90/ <i>tem1</i> , 40/–	LSP 437/00 (1)
	X5	PT6a	60/ <i>spv</i> , 90/–, 40/ <i>tem1</i>	LSP 230/00 (1)
	X6	PT6a	40/ <i>tem1</i>	LSP 524/00 (2)
Blood strains (<i>n</i> =40)	X2	PT1	60/ <i>spv</i>	LSP 233/94 (5)
	X2	PT4	60/ <i>spv</i>	LSP 40/95 (18)
	X2	PT4	60/ <i>spv</i> , 9/ <i>tem1</i>	LSP 79/98 (2)
	X2	PT6	60/ <i>spv</i>	LSP 269/00 (3)
	X2	PT6	60/ <i>spv</i> , others	HCA 91/21937 (1)
	X2	PT6a	60/ <i>spv</i> , 90/–	LSP 11/99 (1)
	X3	PT6a	60/ <i>spv</i> , 40/ <i>tem1</i>	LSP 571/96 (5)
	X7	PNR	67/ <i>spv</i> , 50/–	LSP 108/01 (1)
	X8	PNR	60/ <i>spv</i>	LSP 376/95 (1)
	X9	PT4	–	LSP 421/95 (2)
Control strains	X10	PNR	60/ <i>spv</i>	LSP 141/97 (1)
	X1	PT1	60/ <i>spv</i>	ATCC 13076
	X2	PT4	60/ <i>spv</i>	CNM PT4
	X3	PT6a	60/ <i>spv</i> , 40/ <i>tem1</i>	CNM PT6a

*Phage type, plasmid, and presence of *spv* (conserved region of the *Salmonella* virulence plasmids) and *tem1* (encoding a TEM β-lactamase that confers ampicillin resistance) have been established in previous work (Rodríguez *et al.*, 1998; Soto *et al.*, 2003; unpublished data). –, Unknown function.

†ATCC, American Type Culture Collection; CNM, Centro Nacional de Microbiología (Spain); HCA, Hospital Central de Asturias (Spain); LSP, Laboratorio de Salud Pública del Principado de Asturias.

Table 2. Primers used in this work

Gene target	Location or function	Sequence 5'–3' (forward/reverse)	Amplicon size (bp)	Reference	Accession no.
<i>invE/A</i>	SPI1	TGCCTACAAGCATGAAATGG/AAACTGGACCACGGTACAA	500	Guerra <i>et al.</i> (2000)*	–
<i>orgA</i>	SPI1	GATAAGGCGAAATCGTCAAATG/GTAAGGCCAGTAGCAAAATTG	540	This work	U21676
<i>ttrC</i>	SPI2	GTGGGCGGTACAATATTTCTTTT/TCACGAATAATAATCAGTAGCGC	920	This work	AF282268
<i>ssaQ</i>	SPI2	GAATAGCGAATGAAGAGCGTCC/CATCGTGTTATCCTCTGTCAGC	677	This work	Y09357
<i>mgtC</i>	SPI3	TGACTATCAATGCTCCAGTGAAT/ATTTACTGGCCGCTATGCTGTTG	655	This work	AF106566
<i>misL</i>	SPI3	GACGTTGATAGTCTGCCATCCAG/CAATGCCGCCAGTCTCCGTGC	986	This work	AF106566
<i>spi4R</i>	SPI4	GATATTTATCAGTCTATAACAGC/ATTCTCATCCAGATTGATGTTG	1269	This work	AF060869
<i>spi4D</i>	SPI4	GAATAGAAGACAAAGCGATCATC/GCTTTGTCCACGCCTTTTCATC	1231	This work	AF060869
<i>sopB</i>	SPI5	GATGTGATTAATGAAGAAATGCC/GCAAACCATAAAAACTACACTCA	1170	This work	AE008747
<i>pipA</i>	SPI5	CTCTTGATGATTTTCTTCTT/CTTATCTCAGGCGCGGGTGG	406	This work	AE008747
<i>slyA</i>	Transcriptional regulator	GCCAAAAGCTGAAGCTACAGGTG/CGGCAGGTCAGCGTGTCGTGC	700	Guerra <i>et al.</i> (2000)*	U03842
<i>phoP/Q</i>	Regulatory system	ATGCAAAGCCCCGACCATGACG/GTATCGACCACCACGATGGTT	299	Guerra <i>et al.</i> (2000)*	M25241
<i>agfA</i>	Aggregative fimbriae	TCCGGCCCCGACTCAACG/CAGCGCGGCGTTATACCG	261	Doran <i>et al.</i> (1993)	–
<i>stn</i>	Enterotoxin	TTAGGTTGATGCTTATGATGGACACCC/CGTGATGAATAAAGATAC TCATAGG	617	Guerra <i>et al.</i> (2000)*	–
<i>spvC</i>	Virulence plasmid	ACTCCTTGACACAACCAATGCGGA/TGTCTTCTGCATTTCGCCACC ATCA	424	Guerra <i>et al.</i> (2000)*	517162

*Compiled in this reference, but previously described by different authors.

Diagnostics), 1 µM each primer (Sigma-Genosys), 2 U DyNAzyme II DNA polymerase and amplification buffer (Finnzymes). Amplifications were carried out in a Perkin-Elmer GeneAmp System (Model 9700) using the following program: a hot start cycle of 94 °C for 5 min, then 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, ending with a final extension step of 72 °C for 5 min. Aliquots (15 µl) of the amplification products were analysed by electrophoresis on 2% agarose gels using TAE (Tris-acetate/EDTA) as running buffer (Sambrook & Russel, 2001). Gels were stained with ethidium bromide and visualized with UV light.

Macrorestriction-PFGE and hybridization. Total DNA from *S. Enteritidis* strains was analysed by macrorestriction-PFGE using the CHEF-DRIII SYS220/240 (Bio-Rad) with a standard protocol (Peters *et al.*, 2003). Briefly, *Salmonella* was cultured in LB broth and agarose plugs were prepared with the cells. DNA was digested with *Xba*I (10 U, 4 h, 37 °C; Takara Biomedicals) and the fragments generated were resolved by electrophoresis in 1.2% agarose gels run in 0.5 × TBE (Tris/borate/EDTA) buffer (Sambrook & Russel, 2001). DNA from strains displaying different *Xba*I profiles was also digested with *Bln*I (20 U, overnight, 37 °C; Takara Biomedicals). The running conditions were 200 V at 14 °C for 20 h. The included angle was 120°, and initial and final switch times were 2 s and 64 s, respectively. After electrophoresis the gels were stained with ethidium bromide and visualized with UV light. Relationships among *Xba*I profiles were established on the basis of the number of non-matching bands and the genetic similarity determined by the unweighted pair group method with arithmetic means (UPGMA) and the Jaccard's similarity coefficient (*S*) using the software program MVSP 3.1 (multivariate statistics package for PCs; RockWare).

*Xba*I profiles differing in one or more fragments, and *Bln*I profiles from all strains analysed, were transferred onto membranes and hybridized

with selected probes for the genes indicated, as described by Sambrook & Russel (2001). The DNA probes were obtained by PCR from the type strain *S. Typhimurium* LT2 using the primers displayed in Table 2 and the commercial PCR DIG labelling mix (Roche Diagnostics). Firstly, hybridizations were performed with individual probes, in order to localize the 15 selected genes on specific fragments of the different profiles. Secondly, two combinations of selected probes, mapping on clearly distinguishable fragments, were used to hybridize single membranes. In this way, all hybridization results could be compiled into two panels (see below).

RESULTS

PCR-detection of virulence sequences in clinical strains of *S. Enteritidis*

The 80 clinical and 3 control strains of *S. Enteritidis* (Table 1) were tested for the presence of 15 V genes (10 of them representative of the 5 SPIs, 4 unclustered genes and 1 belonging to pENT, the V plasmid of *S. Enteritidis*) by conventional PCR using the single primer pairs shown in Table 2. Note that for each SPI two primer pairs were assayed, each targeting a specific internal sequence located close to one end of the SPI (Marcus *et al.*, 2000). All strains tested generated the expected amplicons for the 14 chromosomally located V sequences (not shown), showing the presence of the 5 SPIs and the 4 unclustered V genes under study. However, two G strains and two B strains (Table 1) were negative for the plasmid gene *spvC*. In order to simplify

the detection method, several multiplex PCRs, with different combinations of primers, were performed. Among those tested, three combinations (sets I to III, see Methods) were selected, because they generated well-defined profiles and faithfully reproduced the results obtained with individual primers (Fig. 1).

Macrorestriction analysis of *S. Enteritidis* strains

The 83 *S. Enteritidis* strains were first analysed by *Xba*I restriction of the genome, and fragment separation by PFGE. This procedure reveals polymorphisms in the total bacterial DNA, including the chromosome and plasmids larger than 15 kb. A total of 10 different *Xba*I profiles (X1–X10), with fragments ranging from 19 to 900 kb in size (Fig. 2a, b) were found. Each of the control strains generated a distinct profile: X1, X2 and X3 for ATCC 13076, CNM PT4 and CNM PT6a, respectively, while the clinical strains could be distributed into nine profiles (X2–X10), although only X2 and X3, the two prevalent profiles, included both G and B strains (Table 1, Fig. 2a). The 10 profiles shared a total of 9 matching and 16 non-matching bands (some formed by 2 or 3 unresolved fragments). The similarity between profiles was higher for G strains ($\geq 85\%$) than for B strains ($\geq 66\%$).

Interestingly, the X1 profile, only generated by the control ATCC 13076, apparently coincided with the *Xba*I PFGE profile of *S. Enteritidis* SSU7998, for which the *Xba*I genomic cleavage map has been constructed (Liu *et al.*, 1993). Two differences between X1 and the remaining *Xba*I profiles are remarkable: (i) the presence in X1 of 250 and 76 kb fragments (labelled C and G in the SSU7998 *Xba*I restriction map), which were absent from the other nine profiles, and (ii) the presence in the latter of an approximately 265 kb fragment, absent from X1.

Fig. 1. PCR detection of *Salmonella* virulence genes. Gel lane L, DNA ladder (100 bp; Gibco BRL); gel lanes I–III, multiplex PCR using the primer sets I (*spi4D*-1231 bp, *misL*-986 bp, *ssaQ*-677 bp, *orgA*-540 bp, *pipA*-406 bp), II (*spi4R*-1269 bp, *ttrC*-920 bp, *mgtC*-655 bp, *invE/A*-500 bp, *agfA*-261 bp) and III (*sopB*-1170 bp, *slyA*-700 bp, *stn*-617 bp, *spvC*-424 bp, *phoP/Q*-299 bp).

For an accurate mapping of V genes on the *S. Enteritidis* genome (see below), one strain representative of each of the *Xba*I profiles was also subjected to macrorestriction analysis with *Bln*I, a second endonuclease for which the physical map of *S. Enteritidis* SSU7998 is available (Liu *et al.*, 1993). In this case, six different *Bln*I profiles (B1–B6) were detected (Fig. 3a). It should be noted that, although the X1 profile apparently coincided with that of SSU7998, none of the *Bln*I profiles matched exactly the one reported for this strain. In fact, the B1 profile generated by the only X1 strain (ATCC 13076) differed from the *Bln*I profile of SSU7998 (Liu *et al.*, 1993) in that it lacked the F662 fragment of the latter strain, showing instead two fragments of 360 and 300 kb. In addition, it also lacked an approximately 305 kb fragment present in all of the remaining profiles.

Mapping virulence sequences on macrorestriction genomic profiles of *S. Enteritidis*

The mapping of virulence determinants was achieved by Southern blot hybridization of membranes containing the different *Xba*I DNA profiles with probes for the 15 V genes (Fig. 2c, d). Concerning the SPIs, all profiles generated from C and G strains, and all except two profiles (X8 and X10) generated from B strains, carried the (*invE/A-orgA*), (*ttrC-ssaQ*), (*mgtC-misL*), (*spi4R-spi4D*) and (*sopB-pipA*) sequences on matching fragments P670/H656, C250/B245, K170, M300/N286 and A900 (numbers correspond to sizes in kb), respectively. In addition, the probes for SPI2 mapped on a non-matching fragment of 186.5 kb in the X8 profile, and the probes for SPI4 on a non-matching fragment of 485 kb belonging to X10. In both profiles, probes for the remaining islands mapped on the matching fragments indicated above. With regard to other chromosomal V genes: (i) the *slyA* probe mapped apparently on the same fragments as SPI2, (ii) the probes for *phoP/Q* and *agfA* mapped on the approximately 265 kb fragment common to X2–X10, and on one of two alternative fragments [C250 (250 kb) or B245 (245 kb)] in X1, and (iii) the *stn* probe mapped on the same fragments as SPI4 in all profiles except X8, where it was located on a 356 kb fragment. The *spvC* probe mapped on a 60 kb fragment, the size expected for pENT, in seven of the eight *Xba*I profiles generated by strains that proved to be *spvC* positive by PCR amplification. In the remaining profile (X7, only generated by one B strain), *spvC* mapped on a non-matching fragment of approximately 67 kb.

With the data presented above, only SPI3 and SPI5 could be precisely located on defined fragments, labelled K170 and A900 in the SSU7998 *Xba*I map (Liu *et al.*, 1993). Nevertheless, the accurate position of the remaining genes could be established by hybridization of selected probes (*invE/A*, *ssaQ*, *spi4R*, *phoP/Q*, *stn*, *slyA*, *agfA* and *spvC*) with the different *Bln*I profiles (Fig. 3b). In these experiments, (i) the *invE/A* probe (indicative of SPI1) mapped on fragment H684, common to all profiles, (ii) the *ssaQ* (SPI2), *slyA*, *phoP/Q* and *agfA* probes mapped on E595 (which overlaps *Xba*I fragments C250 and B245) in all profiles except B5

Fig. 2. *Xba*I macrorestriction analysis of *S. Enteritidis* and mapping of V genes. (a) PFGE profiles generated from representative strains by *Xba*I digestion: (from left to right) X1, ATCC 13076; X2, CNM PT4; X3, CNM PT6a; X2, LSP 34/00; X3, 49/00; X4, LSP 98/00; X5, LSP 230/00; X6, LSP 524/00; X7, LSP 108/01; X8, LSP 376/95; X9, LSP 421/95; X10, LSP 141/97. A to P (on the left) indicate the fragments identified in the *Xba*I restriction map of SSU7998 (Liu *et al.*, 1993), which coincide with those in ATCC 13076 (X1 profile). (b) Dendrogram of similarity illustrating the genetic relationships between the *Xba*I profiles shown in (a). (c, d) Hybridization of the DNA from (a) with the probes shown on the left. Bold numbers indicate the fragments absent in SSU7998 (Liu *et al.*, 1993). a–f, fragments in which other probes also mapped: a, *pipA*; b, *orgA*; c, *spi4D*; d, *ttrC*, *slyA*; e, *phoP/Q*; f, *mgtC*. L, lambda ladder PFGE Marker (New England BioLabs).

(X8), where they mapped on a non-matching fragment of 562 kb, (iii) the *spi4R* probe (SPI4) mapped on L268 in all except the B6 (X10) profile, where it mapped together with *stn* on a 476 kb fragment, (iv) the *stn* probe mapped on K286 in all of the remaining profiles, except B5 (X8), where it mapped on a 380 kb fragment, and (v) the *spvC* probe gave an identical hybridization pattern with *BlnI* as with *Xba*I. Results of these and the former hybridizations are compiled in Table 3.

DISCUSSION

In this investigation, *S. Enteritidis* strains involved in either gastroenteritis or bacteraemia were screened for virulence factors known to be relevant for pathogenesis in the better

characterized *S. Typhimurium* LT2. By conventional and multiplex PCR, it was demonstrated that the two genes used as indicators for each of five SPIs, as well as four chromosomally located V genes (*slyA*, *phoP/Q*, *agfA* and *stn*), were present in all strains, independently of the outcome of infection. However, the *spvC* gene of pENT was absent from four strains, two causing gastroenteritis (identified as phage type PT6a, carrying a 40 kb *tem1* plasmid and generating X6/B3 macrorestriction profiles) and two involved in bacteraemia (identified as PT4, plasmid free and assigned to X9/B3). It was also shown that a B strain carried an approximately 67 kb *spvC* plasmid. In our laboratory, this gene has been previously detected in the majority of *S. Enteritidis* strains tested, but always as part of the typical 60 kb V plasmid (Rodríguez *et al.*, 1998; Soto *et al.*, 2003).

Fig. 3. *BlnI* macrorestriction analysis of *S. Enteritidis* and mapping of V genes. (a) PFGE profiles generated from representative strains by *BlnI* digestion: (from left to right) B1-X1, ATCC 13076; B2-X2, CNM PT4; B2-X3, CNM PT6a; B2-X2, LSP 34/00; B2-X3, 49/00; B2-X4, LSP 98/00; B2-X5, LSP 230/00; B3-X6, LSP 524/00; B4-X7, LSP 108/01; B5-X8, LSP 376/95; B3-X9, LSP 421/95; B6-X10, LSP 141/97. A to L (on the left) indicate the fragments of the *BlnI* restriction map of SSU7998 (Liu *et al.*, 1993). Fragments labelled 'a' are those not generated by SSU7998, which instead contains a F662 (662 kb) fragment. (b) Hybridization of the DNA from (a) with the probes shown on the left. Bold numbers indicate the fragments absent from SSU7998 (Liu *et al.*, 1993). Fragments labelled 'b' are those in which the *slyA*, *phoP/Q* and *agfA* probes also mapped. L, lambda ladder PFGE Marker (New England Biolabs).

With regard to the applied methodology, the advantage of multiplex PCR versus conventional PCR, for the detection of specific DNA sequences, was clearly shown. Fifteen different V genes could be simultaneously tested by only three PCR reactions. Accordingly, multiplex PCR can be efficiently used to explore the pathogenic potential of a certain bacterium. In further studies, additional multiplex PCR reactions could be designed to screen not only for additional V genes, but also for other genes of interest, such as those involved in antimicrobial drug resistance.

As in other *S. Enteritidis* studies, macrorestriction analysis with *XbaI* and *BlnI* showed a limited genomic heterogeneity in the analysed strains, and identical or similar *XbaI* profiles to those described here have been previously reported in clinical isolates collected in other European (Ridley *et al.*, 1998; Lukinmaa *et al.*, 1999; Garaizar *et al.*, 2000; Peters *et al.*, 2003) and non-European countries (Ahmed *et al.*, 2000; Su *et al.*, 2002; Bakeri *et al.*, 2003). According to similarity coefficients and the number of non-matching fragments, all profiles generated from G strains were closely related, and could then be considered as members of a single *XbaI* restriction cluster ($S \geq 84\%$ and ≤ 4 non-matching fragments). Moreover, all differences observed between profiles of the G group were due to fragments smaller than 100 kb, which could be identified as extrachromosomal DNA. One of them was assigned to pENT, and the other two to R plasmids (as confirmed by hybridization of a *tem1* probe with an approximately 40 kb fragment, common to the X3,

X5 and X6 profiles, and with an approximately 90 kb fragment in the X4 profile; unpublished results). In contrast, the B strains showed a higher DNA polymorphism ($S \geq 66\%$ and ≤ 11 non-matching fragments), and could, therefore, belong to more than one genomic cluster. Additionally, *XbaI* profiles generated by B strains included six non-matching fragments larger than 170 kb, which are probably of chromosomal origin.

It is also noticeable that using the PFGE standard conditions proposed for *XbaI* macrorestriction analysis of *Salmonella* (Sambrook & Russel, 2001), three band groupings present in *S. Enteritidis* profiles (with sizes of 670–656 kb, 330–286 kb and 250–240 kb), in which several virulence probes mapped, cannot be clearly separated. However, well-defined fragments were observed after *BlnI* macrorestriction analysis of strains representative of the different *XbaI* profiles. Taking the physical map of SSU7998 for the *XbaI* and *BlnI* endonucleases as a guide (Liu *et al.*, 1993), hybridization experiments performed here mapped SPI1 to SPI5 on defined fragments of the restriction map of SSU7998 (*XbaI*-H656/*BlnI*-H684, *XbaI*-C255-B245/*BlnI*-E595, *XbaI*-K170, *XbaI*-N300/*BlnI*-L268 and *XbaI*-A900), respectively. They were categorized as matching fragments in most *XbaI* and *BlnI* profiles, including all those generated from G strains. In contrast, probes for SPI2 and SPI4 mapped on non-matching *XbaI* fragments of approximately 186.5 and 485 kb only present in B strains. It is also noticeable that probes for individual V genes mapped together with SPI

Table 3. Mapping of probes for virulence determinants on *Xba*I and *Bln*I macrorestriction profiles of *Salmonella* serovar Enteritidis

The hybridizations are shown in Figs 2 and 3. The distribution of strains in *Xba*I macrorestriction profiles is indicated in Table 1. ND, Not determined.

Probe*	Profile and fragment where the probe mapped†									
	X1/B1	X2/B2	X3/B2	X4/B2	X5/B2	X6/B3	X7/B4	X8/B5	X9/B3	X10/B6
SPI1	H656/H684	H656/H684	H656/H684	H656/H684	H656/H684	H656/H684	H656/H684	H656/H684	H656/H684	H656/H684
SPI2	C250-B245/E595	C250-B245/E595	C250-B245/E595	C250-B245/E595	C250-B245/E595	C250-B245/E595	C250-B245/E595	186·5/562	C250-B245/E595	C250-B245/E595
SPI3‡	K170/ND	K170/ND	K170/ND	K170/ND	K170/ND	K170/ND	K170/ND	K170/ND	K170/ND	K170/ND
SPI4	N300/L268	N300/L268	N300/L268	N300/L268	N300/L268	N300/L268	N300/L268	N300/L268	N300/L268	485/476
SPI5‡	A900/ND	A900/ND	A900/ND	A900/ND	A900/ND	A900/ND	A900/ND	A900/ND	A900/ND	A900/ND
<i>slyA</i>	C250-B245/E595	C250-B245/E595	C250-B245/E595	C250-B245/E595	C250-B245/E595	C250-B245/E595	C250-B245/E595	186·5/562	C250-B245/E595	B245/E595
<i>phoP/Q</i>	C250/E595	265/E595	265/E595	265/E595	265/E595	265/E595	265/E595	265/562	265/E595	265/E595
<i>agfA</i>	C250-B245/E595	265/E595	265/E595	265/E595	265/E595	265/E595	265/E595	265/562	265/E595	265/E595
<i>stn</i>	M286/K286	M286/K286	M286/K286	M286/K286	M286/K286	M286/K286	M286/K286	356/380	M286/K286	485/476
<i>spvC</i> §	60	60	60	60	60	60	67	60	60	60
Representative strain	ATCC 13076	LSP 27/00	LSP 54/00	LSP 98/00	LSP 230/00	LSP 524/00	LSP 108/01	LSP 376/95	LSP 421/95	LSP 141/97

*The probes used for the detection of SPIs are indicated in Methods.

†The fragments labelled with capital letters correspond to the physical map of the strain SSU7998 (Liu *et al.*, 1993).

‡Probes only tested with *Xba*I profiles.

§Marker of pENT (60 kb).

||pENT derivative of 67 kb.

genes on specific fragments. This was for instance the case for *slyA*, *phoP/Q*, *agfA* and SPI2, which were located on the same *BlnI* fragment in all profiles, whereas *stn* and SPI4 mapped on the same or adjacent fragments. In a published work (Collinson *et al.*, 1996b), four fimbriin-encoding genes were mapped on the *XbaI*-*BlnI* genomic restriction maps of two *S. Enteritidis* strains (SSU7998 and 27655-3b) and *S. Typhimurium* LT2. As in the present work, *agfA* was placed on *XbaI* fragments B or C, and on *BlnI* fragment E of SSU7998. More precisely, *agfA* was located between centisomes 40 and 43.3, close to the break point of the 815 kb DNA inversion that distinguishes *S. Enteritidis* from *S. Typhimurium* (Collinson *et al.*, 1996b). According to the results presented here, the *phoP/Q*, *slyA* and SPI2 genes could also be located within the inverted DNA. Within the limits of our mapping experiments, the location of other V determinants roughly coincided in the genome of *S. Enteritidis* and *S. Typhimurium* LT2 (McClelland *et al.*, 2001), thus supporting an identical order in both serovars, as was previously reported for many other genes (Liu *et al.*, 1993).

Taken together, results derived from the present investigation provide the basis for further studies on the genetic background of virulence and the molecular epidemiology of *S. Enteritidis*.

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