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Homologous regions of the *Salmonella enteritidis* virulence plasmid and the chromosome of *Salmonella typhi* encode thiol:disulphide oxidoreductases belonging to the DsbA thioredoxin family

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The nucleotide sequence relatedness between the chromosome of *Salmonella typhi* and the virulence plasmid of *Salmonella enteritidis* was investigated using short DNA probes of < 2 kb covering the whole virulence plasmid sequence. Only one homologous region was detected. This region was subsequently cloned and partially sequenced. Sequences closely related to the *pefI* gene and the ORFs *orf7*, *orf8* and *orf9*, which are located downstream of the fimbrial *pef* operon of the *Salmonella typhimurium* virulence plasmid, were detected. Sequencing of the cloned *S. typhi* DNA fragment also revealed identity with genes of the fimbrial *sef* operon characterized in the chromosome of *S. enteritidis*. These nucleotide sequences mapped upstream of the *S. typhi* chromosomal region homologous to the *S. enteritidis* virulence plasmid. The general organization of the cloned *S. typhi* chromosomal fragment was similar to the fimbriae-encoding region of the *S. typhimurium* virulence plasmid. The deduced product of *orf8* in the *S. typhimurium* virulence plasmid, as well as those of the corresponding ORFs in the homologous region of the *S. typhi* chromosome and in the *S. enteritidis* virulence plasmid (designated *dlt* and *dlp*, respectively), appeared to be related to the thioredoxin family of thiol:disulphide oxidoreductases. The *dlp* gene was able to complement the DTT-sensitive phenotype, the inability to metabolize glucose 1-phosphate and the low alkaline phosphatase activity of a *dsbA* mutant of *Escherichia coli*. The *dlt* gene partially complemented the lack of alkaline phosphatase activity, but not the other mutant phenotypes. The products of both genes could be detected using the T7 RNA polymerase promoter expression system. The estimated molecular masses of the products of the *dlt* and *dlp* genes by SDS-PAGE were 26 and 23 kDa, respectively, the first being in agreement with the deduced amino acid sequence and the latter, somewhat smaller. The processing of a possible leader peptide in the Dlp protein, but not in the Dlt protein, could be responsible for this difference. The Dlp protein appeared as a doublet band on SDS-PAGE, which is characteristic of the oxidized and reduced states of this kind of protein.

Keywords: *Salmonella typhi*, *Salmonella enteritidis*, virulence plasmid, *dsbA*, *dlt* and *dlp*

The EMBL accession numbers for the sequences of *dlt* and *dlp* reported in this paper are X94325 and X94326, respectively.

INTRODUCTION

Several serovars of *Salmonella*, including those most frequently isolated from salmonellosis in humans, harbour virulence-related plasmids, whose role in pathogenesis remains as yet unclear (Gulig *et al.*, 1993). Gulig & Doyle (1993) demonstrated that the virulence plasmid of *Salmonella typhimurium* enhances its growth rate when infecting the mouse. A possible role for the virulence plasmid in systemic infection in humans has been inferred from the higher frequency of plasmid-bearing isolates from blood in comparison to the strains obtained from faeces (Montenegro *et al.*, 1991; Fierer *et al.*, 1992). However, *Salmonella typhi*, the serovar causing typhoid fever in man, lacks a virulence plasmid.

As the virulence plasmid has also been found integrated in the chromosome of *S. typhimurium*, Korpela *et al.* (1989) examined this possibility in *S. typhi* by DNA hybridization. They demonstrated hybridization between a 15 kb fragment of the *S. typhimurium* plasmid and chromosomal DNA of *S. typhi*. However, they did not characterize the homologous regions in either the *S. typhi* chromosome or the virulence plasmid of *S. typhimurium*.

The present study was undertaken to characterize the hybridizing region of the *S. typhi* chromosome. By means of Southern blot hybridization using smaller plasmid-derived DNA probes of < 2 kb, we analysed the nucleotide sequence similarity between the virulence plasmid of *Salmonella enteritidis* strain 82139 (Buisán *et al.*, 1994) and chromosomal DNA of *S. typhi* with similar results to those of Korpela *et al.* (1989). The hybridizing DNA fragment was cloned and sequenced. The translated sequence of one of the identified ORFs showed the motif -Cys-X-X-Cys-, which is present in the active site of the DsbA family of thiol:disulphide oxidoreductase proteins (for reviews see Bardwell, 1994; Loferer & Hennecke, 1994). Enzymes of this type are needed for the secretion and correct folding of diverse proteins, including virulence factors of *Escherichia coli* (Yamanaka *et al.*, 1994; Okamoto *et al.*, 1995), *Vibrio cholerae* (Peek & Taylor, 1992; Yu *et al.*, 1992) and *Shigella flexneri* (Watarai *et al.*, 1995).

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this work are shown in Table 1. *Escherichia coli* strain DH5α was used as the recipient in cloning experiments, with pBluescript SK(+) (Stratagene) as a cloning vector. The plasmid YEp357R was used to obtain translational fusions with a *'lacZ* gene lacking promoter sequences (Myers *et al.*, 1986). All strains were routinely grown at 37 °C in Luria broth (LB) with shaking or on Luria agar plates supplemented, when necessary, with ampicillin (100 μg ml^{-1}), IPTG (50 μg ml^{-1}) and X-Gal (50 μg ml^{-1}). DM minimal salt medium (Neidhardt *et al.*, 1974) supplemented with 10 mM glucose 1-phosphate was used to test the ability to grow using this substrate as sole source of carbon. P medium, containing peptone (Difco; 10 g l^{-1}) and NaCl (5 g l^{-1}) (pH 7·4), was used in the alkaline phosphatase assays (Ito *et al.*, 1983).

Recombinant DNA techniques and nucleotide sequence analysis. Unless otherwise stated, recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). Plasmid DNA was extracted from *Salmonella* strains by the method of Kado & Liu (1981) and from *E. coli* by the procedure of Birnboim & Doly (1979). When necessary for sequencing purposes, plasmid DNA was purified with the Qiagen system. Total DNA from *Salmonella* was obtained as described by Ausubel *et al.* (1993). Southern and colony blot hybridizations were carried out using digoxigenin labelling and chemiluminescent detection systems (Boehringer Mannheim); hybridization reactions were done at 68 °C. DNA sequencing was carried out in an automated laser fluorescent sequencer (ALF, Pharmacia) using the phage M13 universal- and reverse-sequencing fluorescein-labelled primers (Pharmacia). In some cases, sequencing was completed using specific fluorescein-labelled primers synthesized in a Gene Assembler (Pharmacia) and designed to give overlapping reading in both strands. The nucleotide sequences obtained were compiled and analysed using the PC/GENE software (Intelligenetics). The search for similarities in nucleotide and amino acid sequences was done in the EMBL and GenBank databases using FASTA, BLITZ and BLAST software through the Internet.

Alkaline phosphatase assays. Alkaline phosphatase activities were measured essentially as described by Brickman & Beckwith (1975) and Gutierrez *et al.* (1987). Bacterial strains were grown overnight in P medium at 37 °C with shaking. Cells were collected from 1 ml culture by centrifugation and resuspended in 1 ml 10 mM Tris/HCl (pH 8·0), 0·1 M NaCl; 0·5 ml of this suspension was added to 0·5 ml 1 M Tris/HCl (pH 8·0). Then 50 μl 0·1% (w/v) SDS and 50 μl chloroform were added. After agitation, 100 μl 0·4% *p*-nitrophenol phosphate (Sigma) in 1 M Tris/HCl (pH 8·0) was added, and the reaction mixture was incubated at 28 °C until a yellow colour developed. The reaction was then stopped by the addition of 100 μl 1 M KH_2PO_4 and the samples were read in a Beckman DU640 spectrophotometer. Units of alkaline phosphatase were calculated as indicated by Brickman & Beckwith (1975).

DTT sensitivity assays. Complementation of the *dsbA* mutation in *E. coli* strain SS140 was detected by sensitivity to 10 mM DTT, which was the minimum concentration that inhibited the growth of *E. coli* strain SS140 ($DsbA^-$) without affecting growth of *E. coli* wild-type strain CU141 ($DsbA^+$). The assay was done in wells of microtitre plates containing about 8×10^6 cells and 10 mM DTT in 100 μl LB medium. The plate lids were sealed with Teflon sealing tape to prevent DTT evaporation and bacterial growth was observed after 14 h incubation at 37 °C.

Expression of the cloned genes. The T7 phage promoter of plasmid pBluescript SK(+) was used to express the cloned *dlp* and *dlt* genes in *E. coli* strain BL21(DE3) (Table 1). This strain contains a copy of the T7 RNA polymerase gene inserted under the control of the *lacZ* promoter and, therefore, inducible by IPTG. In the presence of rifampicin, the bacterial RNA polymerase is inhibited, and only the genes inserted in plasmid pBluescript downstream of the T7 phage promoter can be expressed (Studier & Moffatt, 1986). The 1·5 kb *NarI–SmaI* fragments of plasmids pST93 and pMJ003 (Fig. 2) were subcloned in pBluescript SK(+) to give rise to the plasmids pST94 (carrying the *dlt* gene) and pSE3 (carrying the *dlp* gene). The expression studies were carried out essentially as described by Sambrook *et al.* (1989), except that 30 min after the induction of the promoter of the T7 RNA polymerase gene with 1 mM IPTG, rifampicin was added to a final

Table 1. Bacterial strains and plasmids used in this work

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
Salmonella enteritidis 82139	Clinical isolate carrying the virulence plasmid	Buisán *et al.* (1994)
Salmonella enteritidis 366	Clinical isolate lacking the virulence plasmid	Ibáñez & Rotger (1993)
Salmonella dublin 19	Clinical isolate carrying the virulence plasmid	Instituto de Salud Carlos III, Madrid*
Salmonella typhi 5866	Clinical isolate from blood sample	Instituto de Salud Carlos III, Madrid*
Salmonella montevideo 6173	Clinical isolate from stool sample	Instituto de Salud Carlos III, Madrid*
Salmonella typhimurium C53	Plasmid-cured derivative of strain C5	Kowarz *et al.* (1994)
Escherichia coli DH5α	*endA1 gyrA96 hsdR17 recA1 relA1 supE44 thi1* Δ*lac*U169 (*ϕ80 lacZ* ΔM15)	Sambrook *et al.* (1989)
Escherichia coli BL21(DE3)	*dcm ompT hsdS gal* (λ c*Its*857 *ind1 Sam7 nin5 lac*UV5-T7 gene 1); a phage λ DE3 lysogen; carries a copy of the T7 RNA polymerase gene under the control of the inducible *lac*UV5 promoter	Studier & Moffatt (1986)
Escherichia coli SS140	*dsbA33*::Tn*5*	Kishigami *et al.* (1995)
Escherichia coli CU141	DsbA$^+$ isogenic parental strain of SS140	Kishigami *et al.* (1995)
Plasmids		
pBluescript SK(+)	pUC19-derived phagemid; encodes ampicillin resistance	Stratagene
YEp357R	pUC18-derived vector suitable for construction of *'lacZ* gene fusions	Myers *et al.* (1986)
pFM82139	61 kb virulence plasmid of the *S. enteritidis* strain 82139	Buisán *et al.* (1994)
pMJ003	Recombinant plasmid containing the 10·3 kb 'C' fragment of pFM82139 inserted in pBluescript SK(+)	Buisán *et al.* (1994)
pSE3	Recombinant plasmid containing the 1·5 kb *Nar*I–*Sma*I fragment of the plasmid pMJ003 inserted in pBluescript SK(+)	This work
pST93	Recombinant plasmid containing a 13 kb fragment of the chromosome of *S. typhi* 5866 inserted in pBluescript SK(+)	This work
pST94	Recombinant plasmid containing the 1·5 kb *Nar*I–*Sma*I fragment of the plasmid pST93 inserted in pBluescript SK(+)	This work

* The Spanish National Centre for Notifiable Diseases.

concentration of 150 μg ml^{-1}, except in the controls. Following 150 min incubation, 1 ml samples of the culture were labelled with 2 μCi (74 kBq) [^{35}S]methionine for 10 min. The cells were collected by centrifugation and suspended in electrophoresis sample buffer. The solubilized proteins were examined by SDS-PAGE using separating and stacking gels containing 15 % (w/v) and 5 % (w/v) acrylamide, respectively.

RESULTS

Detection and cloning of a region from the chromosome of *S. typhi* hybridizing with the *S. enteritidis* virulence plasmid probes

With the aim of detecting the possible relatedness of any region of the chromosome of *S. typhi* with the nucleotide sequence of the virulence plasmid, we used small fragments (< 2 kb) derived from the virulence plasmid of *S. enteritidis* as DNA probes. All the *Hin*dIII fragments obtained from the *S. enteritidis* virulence plasmid pFM82139, which had been previously cloned in plasmid vector pBluescript (Buisán *et al.*, 1994), were further digested with several restriction endonuclease mixtures (*Eco*RI/*Pst*I, *Eco*RI/*Xho*I, *Hae*III/*Hin*dII) or *Hae*III. These fragments (< 2 kb) were labelled with digoxigenin and hybridized against chromosomal DNA from *S. typhi* strain 5866 digested with restriction endonuclease *Bam*HI.

The only DNA fragments of the *S. enteritidis* virulence plasmid which hybridized with chromosomal DNA of *S. typhi* strain 5866 were those contained in recombinant plasmid pMJ003 (Table 1). These DNA probes hybridized with a 13 kb *Bam*HI fragment of *S. typhi* chromosomal DNA (Fig. 1). With the purpose of cloning this fragment, we eluted from a 0·7 % agarose gel the chromosomal fragments of 10–15 kb generated by restriction endonuclease *Bam*HI and ligated them to the cloning vector pBluescript SK(+). Transformants of *E. coli* strain DH5α containing recombinant plasmids were screened by colony blot hybridization using the 5·8 kb *Hin*dIII–*Sma*I DNA probe from plasmid pMJ003 (Fig. 2). One recombinant plasmid which hybridized with this DNA probe, named pST93, was mapped for restriction endonuclease cleavage sites and partially sequenced, as

Fig. 1. Southern blot analysis of chromosomal DNA of *S. typhi* strain 5866 digested with endonuclease *Bam*HI. The DNA fragment of the *S. enteritidis* virulence plasmid contained in the recombinant plasmid pMJ003 was digested with endonuclease *Hae*III, labelled with digoxigenin and used as a DNA probe. The size is indicated on the right in kb.

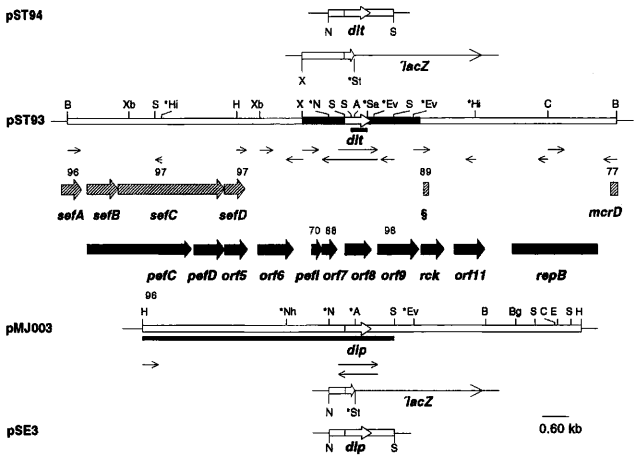

Fig. 2. Linear representation of the constructed plasmids. Outlined bars represent the cloned regions and thin lines the cloning vector. Thin arrows indicate the sequenced regions and the numbers, the percentages of their nucleotide identities with already described genes, which are represented as thick arrows, black for genes of the *S. typhimurium* plasmid (Friedrich *et al.*, 1993) and hatched for chromosomal genes of *Salmonella* (Clouthier *et al.*, 1993, 1994) and *E. coli* (Burland *et al.*, 1995). The region of the chromosome of *S. typhi* strain 5866 homologous to the virulence plasmid of *S. enteritidis* strain 82139 is filled in black; the *dlp* and *dlt* genes are represented by open arrows. §, Non-coding 5′ end of the *sinIM* gene (Karreman & Waard, 1988). Solid bars below plasmids pST93 and pMJ003 indicate the *Sma*I–*Sac*II and *Hin*dIII–*Sma*I fragments, respectively, used as DNA probes. The fusions of *dlt* and *dlp* promoters with the *'lacZ* gene in plasmid YEp357R are also represented. Plasmids pSE3 and pST94 were constructed with the *Nar*I–*Sma*I fragment containing either the *dlp* (from plasmid pMJ003) or the *dlt* (from plasmid pST93) genes, respectively, and used for complementation and protein expression assays. Restriction site abbreviations are as follows: A, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Hi, *Hin*dII; N, *Nar*I; Nh, *Nhe*I; S, *Sma*I; Sa, *Sac*II; St, *Stu*I; X, *Xho*I; Xb, *Xba*I. The sites marked with asterisks can be present in the plasmid more times than indicated.

was the *S. enteritidis* virulence plasmid DNA insert of recombinant plasmid pMJ003 (Fig. 2).

The nucleotide sequence of the cloned *S. typhi* chromosomal region partially corresponds to the fimbrial *sef* genes

Searches in the nucleotide sequence data libraries revealed a high degree of identity (96–97%) among the partial sequences of plasmid pST93 and the *sefA*, *sefC* and *sefD* genes of the *sef* operon previously described in the *S. enteritidis* chromosome, which are required for the production of SEF14 and SEF18 fimbriae (Clouthier *et al.*, 1993, 1994; Collinson *et al.*, 1996).

The region of nucleotide sequence similarity (70–98%) between recombinant plasmid pST93 and plasmid pMJ003 containing the 5·8 kb *Hin*dIII–*Sma*I fragment of the *S. enteritidis* virulence plasmid pFM82139 begins after the *Xho*I site and extends 116 bp beyond the second *Eco*RV site, with a total size of 2·6 kb (Fig. 2). This region of plasmid pMJ003 matches a part of the *pef* locus in the virulence plasmid of *S. typhimurium* that encodes synthesis of fimbriae, namely the ORFs *pefI*, *orf7*, *orf8* and *orf9*, described by Friedrich *et al.* (1993). Nucleotide sequence similarity with the *pefC* gene (96%) was also found adjacent to the left *Hin*dIII site in plasmid pMJ003 (Fig. 2), suggesting that the same organization of this fimbrial operon occurs in both *S. enteritidis* and *S. typhimurium* virulence plasmids (Friedrich *et al.*, 1993). Partial sequencing of plasmid pST93 also showed a substantial sequence similarity to the ORFs *pefI*, *orf7*, *orf8* and *orf9*. As a whole, the genetic organization of the cloned *S. typhi* chromosomal fragment, including the *sef*-related region, was strikingly similar to that of the *pef* region of the *S. typhimurium* plasmid (Friedrich *et al.*, 1993).

The rest (6·2 kb) of the nucleotide sequence of plasmid pST93 did not show significant sequence relatedness to any registered sequence, with the exception of two small regions (Fig. 2). One of them, of 131 bp, is 89% identical to an upstream sequence of the *sinIM* gene of *Salmonella infantis*, which encodes a DNA cytosine methyltransferase (Karreman & Waard, 1988). The second region, of 200 bp, showed 77% identity with the *mcrD* gene, adjacent to the *mcrBC* restriction system genes in *E. coli* but not described in *Salmonella* (Burland *et al.*, 1995).

The *S. enteritidis* virulence plasmid and the homologous locus from the *S. typhi* chromosome could encode a protein related to the DsbA family of disulphide oxidoreductases

No significant relationship between the nucleotide sequence of *orf8* from the virulence plasmid of *S. typhimurium* and the sequences existing in the DNA sequence data libraries has been reported (Friedrich *et al.*, 1993). However, we observed some amino acid sequence similarity between the putative gene product

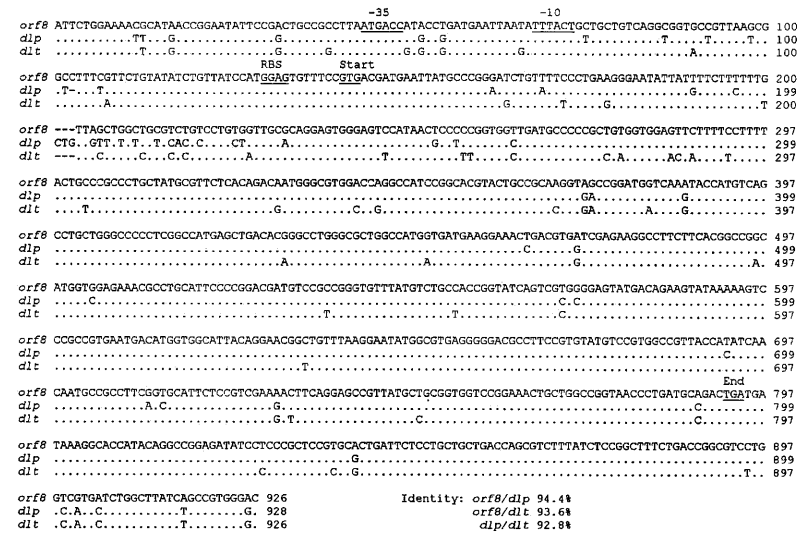

Fig. 3. Comparison of the DNA nucleotide sequences of the DsbA-like encoding ORFs from the plasmids of *S. typhimurium* (*orf8*) (Friedrich *et al.*, 1993) and *S. enteritidis* (*dlp*), and from the chromosome of *S. typhi* (*dlt*). Only the changes in the nucleotide sequences of the *dlp* and *dlt* genes are shown; gaps introduced by the program to optimize the alignment are represented by dashes. The possible −35 and −10 regions, ribosome-binding site (RBS) and the start and stop codons are underlined, according to the published data (Friedrich *et al.*, 1993).

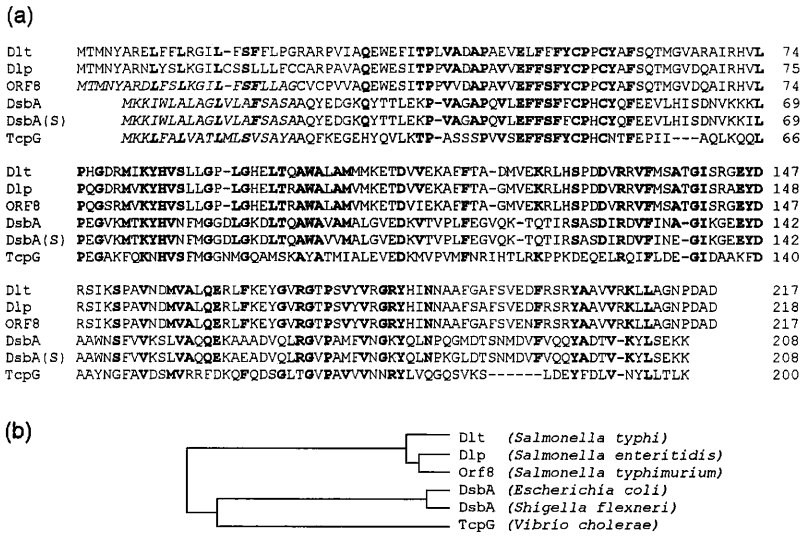

Fig. 4. (a) Alignment of the translated amino acid sequences of the products of *orf8* (Friedrich *et al.*, 1993) and the *dlt* and *dlp* genes (named, respectively, ORF8, Dlt and Dlp) with the published sequences of the DsbA proteins from *E. coli* (Bardwell *et al.*, 1991) and *Sh. flexneri* [here named DsbA(S); EMBL accession no. D38253], and of the TcpG protein from *V. cholerae* (Peek & Taylor, 1992). Amino acids that are identical in more than three sequences are emphasized in bold; gaps introduced by the program to optimize the alignment are represented by dashes. The predicted leader peptides appear in italics. (b) Dendrogram of similarity of the amino acid sequences, calculated with the CLUSTAL software of PC/GENE (Intelligenetics).

of the translated *orf8* ORF and the members of the DsbA family of thiol:disulphide oxidoreductases, mainly around the preserved motif Cys-X-X-Cys, which has been reported to be essential in this type of enzyme (for a review see Bardwell, 1994). The deduced amino acid sequences of the putative products of the corresponding ORFs in the cloned homologous fragments of the *S. typhi* chromosome (plasmid pST93) and the virulence plasmid of *S. enteritidis* (plasmid pMJ003) exhibited the same similarity to the DsbA family of enzymes. We named these ORFs *dlt* and *dlp*, respectively. The alignments of the nucleotide and amino acid sequences corresponding to these genes and those of the other bacteria are shown in Figs 3 and 4.

In order to clarify whether these ORFs could actually encode disulphide oxidoreductase enzymes, we tried to complement several defective phenotypes of *E. coli* strain SS140 (DsbA$^-$) (Kishigami *et al.*, 1995). Plasmids pMJ003 or pSE3 or both, each of which contains the *dlp* gene (Fig. 2), were able to restore the wild-type phenotypes of *E. coli* strain CU141 (Table 2). On the other hand, plasmid pST93 or its subclone pST94 or both, each of which contains the *dlt* gene (Fig. 2), partially complemented the lack of alkaline phosphatase activity but failed to restore the resistance to 10 mM DTT and the ability to grow on glucose 1-phosphate as sole source of carbon (Table 2).

As the failure of the *dlt* gene to efficiently complement the phenotype of the DsbA$^-$ strain of *E. coli* could have been due to a lack of expression, we constructed gene fusions of both the *dlp* and *dlt* genes with a promoter-deficient *'lacZ* gene (Fig. 2). Similar *β*-galactosidase activities on X-Gal plates were detected in both cases. The products of the *dlp* and *dlt* genes were detected after their overexpression using the T7 phage promoter (Fig. 5). The product of the *dlt* gene is a protein of 26 kDa, a

Table 2. Complementation of the *E. coli dsbA* mutation with plasmids carrying the chromosomal *S. typhi dlt* gene or the *S. enteritidis* virulence plasmid *dlp* gene

Strain/plasmid	Growth with 10 mM DTT*	Growth on glucose 1-phosphate medium†	Alkaline phosphatase‡
CU141 (*dsbA*$^+$)	+	+	100·0%
SS140 (*dsbA* mutant)	−	−	42·5%
SS140/pBluescript SK(+)	−	−	42·6%
SS140/pMJ003 (*dlp*$^+$)	+	+	122·0%
SS140/pSE3 (*dlp*$^+$)	+	ND	102·6%
SS140/pST93 (*dlt*$^+$)	−	−	78·8%
SS140/pST94 (*dlt*$^+$)	−	ND	83·8%

ND, Not determined.

* Resistance to DTT was assayed in LB medium with 10 mM DTT.

† Ability to grow with glucose 1-phosphate as the sole source of carbon was assayed in DM minimal medium with 10 mM glucose 1-phosphate.

‡ Alkaline phosphatase activities were determined by the method of Brickman & Beckwith (1975) and expressed as a percentage of the activity detected in *E. coli* strain CU141, to which was assigned a value of 100%.

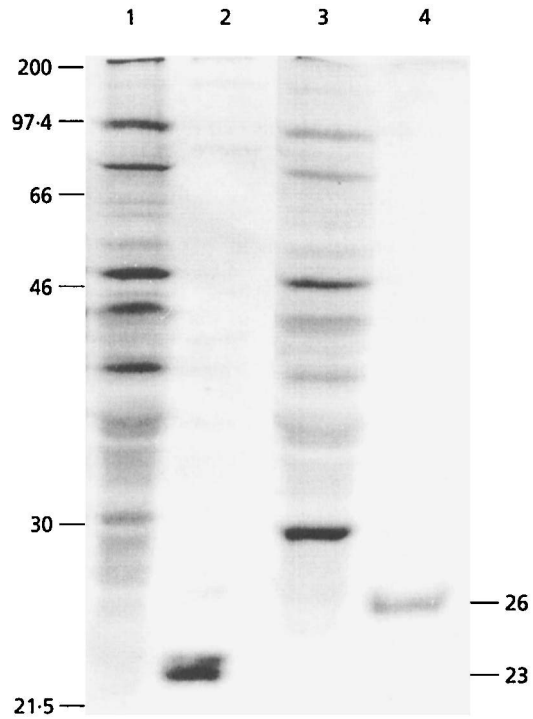

Fig. 5. Expression of the *dlp* and *dlt* genes under the influence of the T7 phage promoter. An autoradiogram of the dried gel is shown; numbers on the left indicate the positions and molecular masses in kDa of the pre-stained protein molecular size standards (Amersham). Lanes 1 and 2 correspond to cell extracts of *E. coli* strain BL21(DE3) containing the plasmid pSE3 (*dlp*) and lanes 3 and 4 correspond to cell extracts of *E. coli* carrying the plasmid pST94 (*dlt*; see Fig. 2). The bacteria corresponding to lanes 1 and 3 were not treated with rifampicin.

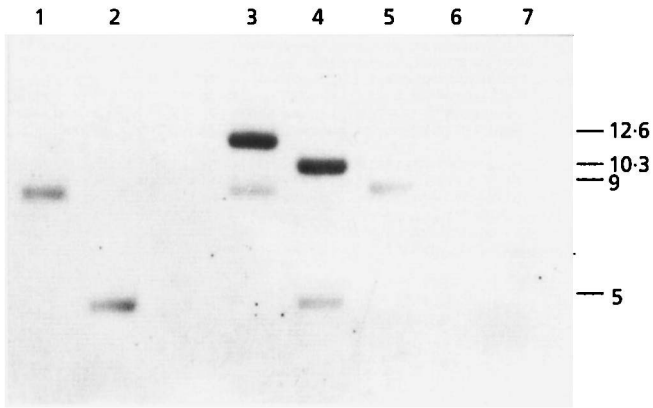

Fig. 6. Southern blot analysis of total DNA isolated from the following strains and digested either with restriction endonucleases *Bam*HI (lanes 1, 3, 5, 6 and 7) or *Hin*dIII (lanes 2 and 4): *S. enteritidis* strain 366 (lanes 1 and 2), *S. enteritidis* strain 82139 (lanes 3 and 4), *S. dublin* strain 19 (lane 5), *S. typhimurium* strain C53 (lane 6) and *S. montevideo* strain 6173 (lane 7). The DNA was transferred to a nylon membrane and hybridized with the 409 bp *Sma*I–*Sac*II fragment of plasmid pST93 (see Fig. 2) labelled with digoxigenin as a probe. Sizes are indicated on the right in kb.

molecular mass that agrees with that deduced from the amino acid sequence of the gene product (24700 Da). The expression of the *dlp* gene consistently gave two adjacent protein bands on SDS-PAGE. This observation agrees with the reported data about the DsbA protein of *E. coli*, which also gave two bands in SDS-PAGE corresponding to the oxidized and reduced states of the cysteine residues (Kishigami *et al.*, 1995). The estimated

molecular mass in SDS-PAGE of the Dlp protein was about 23 kDa, whereas that deduced from the amino acid sequence of the gene product was 24400 Da.

Presence of *dlp*-related sequences in the chromosome of other serotypes of *Salmonella*

Some *Salmonella* serovars were examined by Southern blot hybridization using the 409 bp *Sma*I–*Sac*II fragment of plasmid pST93 as a DNA probe (Fig. 2). We detected hybridization with chromosomal DNA fragments of 9 or 5 kb (corresponding to the digestion with endonucleases *Bam*HI or *Hin*dIII, respectively) from *S. enteritidis* strain 366, which lacks the virulence plasmid, and *Salmonella dublin* strain 19, which harbours a virulence plasmid (Fig. 6). Hybridizing fragments of the same size were detected in total DNA from *S. enteritidis* strain 82139, together with others (of 12·6 and 10·3 kb, corresponding to the digestion with *Bam*HI or *Hin*dIII, respectively) which should belong to the virulence plasmid. It is noteworthy that neither the plasmid-cured strain C53 of *S. typhimurium*, nor *Salmonella montevideo* strain 6173, yielded hybridization reactions (Fig. 6).

DISCUSSION

The lack of a virulence plasmid in *S. typhi* is striking, considering its essential role in systemic infections caused by other serovars. However, most of the plasmid-borne genes, except the *spv* operon, are of marginal or unknown importance in pathogenesis. The finding of virulence-plasmid-related genes in the chromosome of *S. typhi* could be indicative of the importance of their roles. However, we did not find homologous regions additional to that reported by Korpela *et al.* (1989), despite the use of shorter plasmid-derived DNA probes which should have allowed us to detect single genes.

Analysis of the DNA fragments cloned from the chromosome of *S. typhi* and from the plasmid of *S. enteritidis* showed that the homologous regions were adjacent to fimbriae-encoding regions in these strains. The present report presents the first direct evidence of the presence and organization of the *sefA*, *sefC* and *sefD* genes in *S. typhi*. The structural subunit of the SEF18 fimbriae, which is encoded by the *sefD* gene, was previously detected immunologically in *S. typhi*, but there is no evidence of the functionality of these fimbriae as adhesins (Clouthier *et al.*, 1994). In the case of the SEF14 fimbriae, the *sefA* gene, which encodes their structural subunit, was previously detected by DNA hybridization in *S. typhi*, but its expression could not be confirmed by agglutination tests (Turcotte & Woodward, 1993). Therefore, the role of these genes in the adhesion of *S. typhi* remains unproven.

The similarity of its deduced amino acid sequence, and its complementation of a *dsbA* mutant of *E. coli*, allows us to propose the inclusion of the product of the *dlp* gene in the thioredoxin family of thiol:disulphide oxidoreductases (Bardwell, 1994; Loferer & Hennecke, 1994). The variable degree of complementation of the *dsbA* mutation with the *dlt* gene could be explained by a different specificity or activity of the enzyme, or by a different cellular localization. It is known that the DsbB protein is required in *E. coli* for the reoxidation of DsbA (Bardwell, 1994), but the oxidoreduction system could be different in *S. typhi*. The lack of visualization of the putative oxidized and reduced forms of the Dlt protein (Fig. 5) could support the latter hypothesis, but it would be necessary to know the actual oxidoreduction state of the protein. The estimated molecular mass of the Dlp protein of 23 kDa is as would be expected after processing of a signal peptide, as occurs with other DsbA-like proteins. However, the deduced amino acid sequences of the Dlp and Dlt proteins clearly differ from those of the DsbA proteins of *E. coli*, *Sh. flexneri* and *V. cholerae* in the region corresponding to the leader peptide (Fig. 4). Moreover, we could not detect a consensus sequence for a signal peptidase processing site either in the Dlt protein or in the Dlp protein. The deduced amino acid sequence of the amino-terminal region of the Dlp and Dlt proteins appears to be much more similar to that of the *orf8* gene product than to those of the other DsbA proteins (Fig. 4). It has been reported than the deduced amino acid sequence of the *orf8* gene product of the virulence plasmid of *S. typhimurium* reveals a signal sequence of 21–23 amino acids typical of a lipoprotein (Friedrich *et al.*, 1993). It is tempting to explain the differences in molecular masses and in activities of the Dlp and Dlt proteins by the lack of signal peptide processing in the latter protein; in fact, the deduced amino acid sequence of the Dlp protein is much more closely related to that deduced from *orf8* around the predicted cleavage site than to the Dlt amino acid sequence (Fig. 4).

The proximity of the *dlp* and *dlt* genes to the fimbrial operons *pef* and *sef* could suggest a function of these genes in the processing of some component of these fimbriae. Moreover, we did not detect a reaction with a *dlt* gene probe in the two strains of *Salmonella* belonging to serogroups other than serogroup D (namely *S. typhimurium* and *S. montevideo*), in agreement with the report of the presence of *sefA* only in members of serogroup D (Turcotte & Woodward, 1993; Collinson *et al.*, 1996). To our knowledge, there is no information about the presence of the *pef* operon in the virulence plasmid of *S. dublin*, and we did not detect apparent hybridization of the *dlt* gene probe with DNA of this plasmid (Fig. 6). The hybridization of the *dlt* gene probe with DNA of the *S. enteritidis* chromosome indicates that this serovar could have a second gene encoding thiol:disulphide oxidoreductase associated with the *sef* operon. The synthesis of Pef fimbriae is independent of ORFs 7, 8, 9 and 11, but the adhesiveness of these fimbriae could not be proved (Friedrich *et al.*, 1993); in the same way, the correct assembly of SEF14 and SEF18 fimbriae appears to be dependent on some unknown

accessory genes (Clouthier *et al.*, 1993, 1994). It should also be borne in mind that the expression of these fimbriae has always been performed in a DsbA$^+$ background, which could disguise the need for expression of the *dlt* or *dlp* genes or of *orf8*. It is noteworthy that the nucleotide sequence identity between plasmid pST93 and the *pef* operon includes *pefI*, the putative regulator (Friedrich *et al.*, 1993), which suggests that an undescribed regulatory gene of the chromosomal *sef* operon could also exist. These possibilities are currently under investigation.

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