Genomic analysis and growth-phase-dependent regulation of the SEF14 fimbriae of Salmonella enterica serovar Enteritidis

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Salmonella enterica serovar Enteritidis is a leading cause of food poisoning in the USA and Europe. Although Salmonella serovars share many fimbrial operons, a few fimbriae are limited to specific Salmonella serovars. SEF14 fimbriae are restricted to group D Salmonella and the genes encoding this virulence factor were acquired relatively recently. Genomic, genetic and gene expression studies have been integrated to investigate the ancestry, regulation and expression of the sef genes. Genomic comparisons of the Salmonella serovars sequenced revealed that the sef operon is inserted in leuX in Salmonella Enteritidis, Salmonella Paratyphi and Salmonella Typhi, and revealed the presence of a previously unidentified 25 kb pathogenicity island in Salmonella Typhimurium at this location. Salmonella Enteritidis contains a region of homology between the Salmonella virulence plasmid and the chromosome downstream of the sef operon. The sef operon itself consists of four co-transcribed genes, sefABCD, and adjacent to sefD there is an AraC-like transcriptional activator that is required for expression of the sef genes. Expression of the sef genes was optimal during growth in late exponential phase and was repressed during stationary phase. The regulation was coordinated by the RpoS sigma factor.

Keywords: pili, salmonellosis, regulation, genomics, pathogenicity islands

INTRODUCTION

Fimbrial adhesins mediate interactions between bacteria and solid surfaces, including attachment to host cells. Among the pathogenic enteric bacteria multiple types of fimbriae are essential for a virulent lifestyle. For example, Salmonella possess fimbriae that bind to different host tissues, either intestinal epithelial tissues, specific intestinal cells involved in the immune response (M-cells) or macrophages (Bäumler et al., 1996a, b; Edwards et al., 2000). Fimbriae with limited distribution and specific roles in virulence are thought to contribute to some of the unique aspects of virulence not seen in closely related bacteria.

Most fimbriae (with the notable exception of the type-IV pili) have a similar mechanism of translocation and biogenesis. The fimbrial shaft is composed of a major subunit that is translocated across the cytoplasmic membrane via the general secretory (sec-dependent) system. In the periplasm the subunits are prevented from premature aggregation by binding to a chaperone. Resolution of the crystal structure of two fimbrial chaperone-subunit complexes elegantly revealed the interactions involved in regulating the periplasmic embrace between these two partners, simultaneously preventing premature subunit–subunit interactions but allowing subunit translocation across the outer membrane (Choudhury et al., 1999; Sauer et al., 1999). A specific usher protein mediates export across the outer membrane, with a distinct chaperone–usher pair encoded by each fimbrial operon. Although chaperones and ushers from different fimbrial systems are very homologous, the usher and chaperone work together and are normally specific for the fimbrial subunits – a chaperone and usher pair will not usually mediate translocation of fimbrial subunits from another operon.

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Many fimbrial systems also encode one or more minor components (so-called because they are much less abundant than the major subunit) that are exported as part of the fimbrial structure (Edwards et al., 1996; Edwards & Puente, 1998). These subunits often form the adhesin that mediates the interaction between the fimbriae and the solid surface (Khan & Schifferli, 1994). A variety of fimbriae have previously been characterized in *Salmonella enterica*, including the plasmid-encoded (PEF), long polar (LPF), thin aggregative (AGF) and type I (FIM) fimbriae (Bäumler et al., 1997; Townsend et al., 2001). Many of the fimbriae from *Salmonella enterica* subsp. *enterica* serovar Enteritidis are similar to fimbriae from *Salmonella* Typhimurium, although a number of these fimbriae were initially given alternate names (for example the *Salmonella* Enteritidis AGF homologue was initially called SEF17 and the FIM homologue was initially called SEF21). In addition to the fimbriae that are shared between serovars, *Salmonella* Enteritidis contains fimbriae such as the *Salmonella* Enteritidis SEF14 fimbriae that are not found in *Salmonella* Typhimurium. The SEF14 fimbriae require four proteins for biogenesis: the major subunit (SefA), chaperone (SefB) and usher (SefC) have previously been characterized (Clouthier et al., 1993; Thorns et al., 1990; Turcotte & Woodward, 1993). The minor subunit (SefD) was also previously identified but not thought to be part of the SEF14 fimbriae (Clouthier et al., 1994).

We recently reported the role of the SEF14 fimbriae in virulence and showed that SEF14 fimbriae are essential for binding to macrophages (Edwards et al., 2000). In this report we describe a multi-faceted approach to dissecting the sef operon, utilizing genomics and genetics to define and inactivate genes. We identify the principal regulatory element that controls sef gene expression and characterize environmental regulation of sef gene expression.

**METHODS**

**DNA analysis.** The *Salmonella* Typhi genomic DNA sequence was obtained from the Sanger Centre (http://www.sanger.ac.uk; J Parkhill, unpublished), the *Salmonella* Typhimurium and *Salmonella* Paratyphi genomic DNA sequences were obtained from the Washington University St Louis Sequencing Center (http://genome.wustl.edu/; M. McClelland, unpublished) and the *Salmonella* Enteritidis genome is currently being sequenced at the University of Illinois (R. A. Edwards, D. N. Green, G. J. Olsen & S. R. Maloy, unpublished). DNA sequence from the *Salmonella* Enteritidis project and further information about the genome sequencing projects is available from http://www.salmonella.org. BLAST searches were performed either via the NCBI website (http://www.ncbi.nlm.nih.gov) or locally using the WU-BLAST executables (Altschul et al., 1990, 1994; Altschul & Gish, 1996; http://blast.wustl.edu). *Salmonella* Enteritidis DNA was sequenced either from clones (described below) or directly from PCR products. Both types of samples were sequenced using ABI big-dye termination sequencing by the University of Illinois Biotechnology Center (http://www.life.uiuc.edu/biotech). To generate PCR products, primers were designed to be identical (except for the introduction of restriction endonuclease sites) to either the known *Salmonella* Enteritidis sequence (where available) or to the corresponding sequence in *Salmonella* Typhi or *Salmonella* Typhimurium as appropriate. PCR was performed as described elsewhere (Maloy et al., 1996) using annealing temperatures calculated from the G+C content of the primers. PCR products were analysed by gel electrophoresis and purified using the Wizard PCR purification kit (Promega) prior to sequencing.

**Bacterial strains, plasmids, phage and growth conditions.** All bacterial strains, plasmids and phage used in this study are given in Table 1. All strains were grown in Luria–Bertani (LB) medium, supplemented with the following antibiotics where necessary (final concentrations, µg/ml): kanamycin, 50; chloramphenicol, 30; tetracycline, 10; ampicillin, 90. All transductions were performed using P22 HT int. Transductants were purified on Evans Blue Uranine (EBU) plates and checked for lysogens by cross-streaking against phage P22 H5 as described previously (Maloy et al., 1996).

**Strain construction.** To facilitate genetic manipulation sefD was amplified by PCR and cloned into the ∼ dependent suicide vector pDMS197. The ∼ dependent plasmids were routinely maintained in MST4718, a ∼ derivative of wild-type *Salmonella* Typhimurium strain LT2 (A. E. Stanley & S. R. Maloy, unpublished). To construct lacZ ooper fusions between sefD and MudJ, MST4718(pRE204) was used as a recipient in transductions with phage P22 grown on TT10289 (Maloy et al., 1996). Transitory cis-complementation of the transposase-deficient MudJ allows random insertion events, a fraction of which will be in the plasmid. Potential fusions were identified by the co-localization of TetR from pDMS197 and KanR from MudJ on the same plasmid. Transposon insertions with MudJ fused to sefD were identified by PCR using one primer that hybridizes in SefD (5′-GGTCAAGATCTTCACCCATTATTACATCAG-3′) and a second primer that hybridized in the trpAB region of MudJ (5′-CAGAGATCGCATCGACTCGACCGCT-3′). The PCR products were sequenced to confirm the exact point of insertion of the MudJ element.

To introduce the mutations onto the chromosome, the suicide plasmid was electroporated either into TYT3362 (a sefR derivative of *Salmonella* Typhimurium LT2 whose construction is described below) or wild-type L5K with selection for the MudJ-encoded KanR marker. Enrichment for second recombination events that eliminated the suicide vector was performed by growth on sucrose (Edwards et al., 1998) and confirmed by checking for loss of the plasmid-encoded TetR marker and by PCR to confirm inheritance of the mutant allele.

To construct both the constitutively expressed sefR construct (pRFE234) and the fusion between sefR and the arabinose-inducible promoter (pRFE235), a sefR PCR product was cloned into either pUC19 or pBAD18, respectively, using primers 5′-GGTCAAGATCTTCACCCATTATTACATCAG-3′ and 5′-CGGAGTTCTGAGTGAGGGTGAAAT-3′. This 1078 bp product encodes the 3′ end of the sefD gene, all of the sefR gene and 38 bp upstream of sefR. The expression of sefR in the pUC19 clone is controlled by the lac promoter in this plasmid, and the pBAD18 construct contains the arabinose-inducible promoter and araC, the gene encoding the transcriptional activator for expression from that promoter (Guzman et al., 1995).

**Identification of linked markers and genetic manipulations.** To identify markers linked to the sef island, plasmid pBSL142 (a delivery vehicle for TN5dGen; Alexeyev et al., 1995) was electroporated into *Salmonella* Enteritidis sefA1::Kan that had been heated to inactivate the restriction modification system (Edwards et al., 1999). Because pBSL142 is a suicide plasmid, any GenR colonies should result from transposition.
Typhimurium LT2. The transductants were screened for the operon, but not within the operon itself co-transduces with One of the Tn5dGen mutations located very close to the sefA1 allele that lies outside of the sef island and has no effect on the regulation of expression of the sefD::MudJ fusion. The markers were moved into Salmonella Enteritidis LT2 by transduction followed by selection for GenK and screening for both sefA1::Kan and sefD::MudJ by PCR. The presence of sefB and lacZ were confirmed by PCR both before and after expression studies to confirm that the sefA1::Kan and MudJ elements did not recombine. If the sefA1::Kan and sefD::MudJ elements recombined both sefB and lacZ would be deleted. Although this may happen at a very low frequency, such recombination events were never observed.

One of the Tn5dGen mutations located very close to the sef operon, but not within the operon itself co-transduces with sefA::Kan approximately 80% of the time. This insertion does not affect virulence and lies outside the sef insertion in the genome. This transposon insertion was used to backcross the ΔsefA mutation into an otherwise wild-type background by isolation of a strain with the transposon linked to the in-frame deletion, followed by P22 transductions of the transposon into LK5 and screening for co-inheritance of the ΔsefA allele.

**β-Galactosidase assays.** Assays were performed as described elsewhere and the activities are reported in Slauch units (Maloy, 1990; Slauch & Silhavy, 1991).

**RESULTS**

**Analysis of the sef operon**

The sef fimbrial operon (Fig. 1) contains all the genes required for biogenesis of the SEF14 fimbriae. sefA encodes the most abundant subunit of the fimbrial shaft and sefD possibly encodes the tip-located adhesin (R. Edwards, unpublished). Based on the high level of sequence homology with previously characterized fimbrial proteins, the proteins encoded by sefB and sefC are the periplasmic-located chaperone and outer-membrane-located usher, respectively. We show below
that the fifth \textit{sef} gene, \textit{sefR}, encodes a transcriptional regulator of the \textit{sef} operon. The \textit{dlp} gene located downstream of \textit{sefR} encodes a thiol-disulphide oxidoreductase that is a homologue of \textit{dbsA}. This gene was initially identified as a homologous region between the \textit{Salmonella} Enteritidis virulence plasmid and the \textit{Salmonella} Typhi genome (Rodriguez-Pena \textit{et al.}, 1996). As \textit{Salmonella} Typhi does not contain the virulence plasmid, the \textit{Salmonella} Typhi \textit{dlp} homologue (called \textit{dlt}) must be located on the chromosome in this serovar. Analysis of the \textit{Salmonella} Typhimurium genome sequence suggests that the only \textit{dlp} homologue present in this serovar is located on the virulence plasmid adjacent to a different fimbrial operon (\textit{pef}). A region that extends downstream from \textit{Salmonella} Enteritidis \textit{sefD} was PCR-amplified and sequenced, and showed that both \textit{sef} and \textit{dlp} are located on the chromosome in \textit{Salmonella} Enteritidis. Furthermore, our genome sequencing has confirmed the chromosomal location of \textit{sef} in \textit{Salmonella} Enteritidis. We have sequenced the entire virulence plasmid, including the \textit{sef} region and the \textit{dlp} homologue adjacent to this, and have not identified any virulence plasmid-encoded \textit{sef} sequences. In \textit{Salmonella} Enteritidis alone, \textit{dlp} is on both the chromosome and the virulence plasmid.

\textbf{Genomic comparisons}

The \textit{sef} island was initially mapped to 97.6 centisomes (\textit{cs}) on the \textit{Salmonella} Enteritidis chromosome (Collinson \textit{et al.}, 1996). The order of genes surrounding the \textit{sef} island – \textit{valS} (97.3 \textit{cs}), \textit{pepA} (97.4 \textit{cs}), \textit{leuX} (previously unmapped), \textit{sef}, \textit{dlp} (previously unmapped), \textit{mcrD} (previously unmapped) and \textit{trpRS} (99.9 \textit{cs}) – confirmed this observation (Sanderson \textit{et al.}, 1996). From the comparison of several closely related sequences, the \textit{sef} insertion is located within the \textit{leuX} gene, which encodes a leucine-tRNA. The \textit{sef} island has a mean G + C content of 35.4 mol\%, significantly less than the mean for the \textit{Salmonella} chromosome of 51.9 mol\%. By comparing the G + C content profile the junctions of the \textit{sef} operon with the ancestral chromosome could be identified. The junction adjacent to \textit{sefA} occurs at an IS1230 element; however, there does not appear to be any ancestral insertion scars in the genomic sequence at the \textit{sefR} end of the island. The \textit{sef} insertion \textit{per se} is 6751 bp, extended from 779 bases upstream of \textit{sefA} to 926 bases downstream of \textit{sefR} (data not shown). This analysis suggests that \textit{dlp} is not part of the \textit{sef} island and may have been acquired independently.

Three group D \textit{Salmonella} serovars have been shown to contain the \textit{sef} operon inserted at \textit{leuX}: \textit{Salmonella} Enteritidis, \textit{Salmonella} Typhi and \textit{Salmonella} Paratyphi. Phage genomes separate \textit{leuX} and the IS1230 element/\textit{sef} island in the latter two serovars, but not in \textit{Salmonella} Enteritidis (Fig. 1). Sequencing data suggests that the \textit{Salmonella} Typhi \textit{sef} operon contains multiple frameshift mutations. These have been confirmed by independent sequencing, suggesting that the \textit{sef} genes are not expressed in \textit{Salmonella} Typhi (Townsend \textit{et al.}, 2001).

\begin{figure}[ht]
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\caption{Organization of the \textit{sef} gene cluster and adjacent DNA. The \textit{sef} gene cluster lies between \textit{leuX} and \textit{trpS}. In \textit{Salmonella} Enteritidis the five \textit{sef} genes are separated from \textit{leuX} by a single insertion element. In \textit{Salmonella} Typhi a complete P4 phage (approx. 11.5 kb) is inserted at \textit{leuX}. This phage is absent from \textit{Salmonella} Enteritidis. There are no \textit{sef} genes in \textit{Salmonella} Typhimurium, but this position on the chromosome is occupied with a previously unidentified and uncharacterized pathogenicity island. A single ORF on this island has been identified by random mutagenesis to be induced when bacteria are internalized by macrophages (Pfeifer \textit{et al.}, 1999). (a) Schematic organization of the region surrounding the \textit{sef} insertion. (b) Organization of the \textit{sef} operon.}
\end{figure}
Unlike *Salmonella* Typhi and *Salmonella* Typhimurium, there is no phage genome inserted between the IS1230 element and *leuX* in *Salmonella* Enteritidis. However, the entire *leuX* gene has been substituted by a putative transposon containing a different *leuX* gene. The transposon contains 16 bp direct inverted repeats that delimit the foreign DNA inserted into the *Salmonella* Enteritidis genome and a transposase gene immediately adjacent to one inverted repeat (Fig. 1).

*Salmonella* Typhimurium, which is not a member of the group D *Salmonella*, does not contain any of the *sef* island, IS1230 element, transposon or phage genome at *leuX*. Based upon analysis of the *Salmonella* Typhimurium genome sequence, we have identified a previously uncharacterized 25337 bp pathogenicity island inserted at this site (data not shown). This island contains very limited homology to sequences in the databases; however, one ORF is similar to the *Shigella flexneri* virulence gene, *shiB*, and another was identified as being specifically induced inside macrophages (Pfeifer et al., 1999). This island has not been characterized further; however, it indicates that as in *Escherichia coli* there have been multiple independent acquisitions of pathogenicity islands at *leuX* in the *Salmonella* chromosome: the *sef* island was probably inherited after group D *Salmonella* diverged from other groups and *Salmonella* Typhimurium inherited a separate island.

The sequence of the *Salmonella* Enteritidis *sef* region from more than 1 kb upstream of *sefA* to a few base pairs downstream of *sefD* was available from GenBank as several different sequences (accession nos L11008, L11009, L11010, U07129 and X98516); however, sequence information in the region downstream of *sefD* was not available. Using primers designed to be compatible with *Salmonella* Enteritidis *sefD* and *Salmonella* Typhi *mcrD* regions, the DNA downstream of *sefD* was PCR-amplified and sequenced. The sequence adjacent to *sefD* in *Salmonella* Enteritidis was similar to the corresponding region from the *Salmonella* Typhi genome and contains both *sefR*, encoding the transcriptional activator, and *dlp*, encoding the thiol-disulphide oxidoreductase. The sequence was compiled together with the previously published sequences and the entire contig was deposited in the GenBank database with the accession number AF239978. The sequence of adjacent regions in the *Salmonella* Enteritidis genome have been released as part of the genome sequencing project and can be retrieved from http://www.salmonella.org.

**Transduction of the *sef* island to *Salmonella* Typhimurium LT2**

A genetic approach was taken to characterize the islands and genes that were identified from the genomic analysis. *Salmonella* Enteritidis contains a potent restriction system that limits genetic manipulations in this strain. The restriction system was circumvented by moving the entire *sef* island from *Salmonella* Enteritidis to *Salmonella* Typhimurium. Several Tn5dGen transposon insertions linked to the *sef* operon were identified. Following transduction from *Salmonella* Enteritidis to *Salmonella* Typhimurium two classes of insertion were found. The first were 100% linked to the *sefA* operon in transductions between *Salmonella* Enteritidis and

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**Fig. 2.** Transduction of *sef*-linked markers. The *sef* genes are on an island not found in *Salmonella Typhimurium*. Linked transposons can either lie within the island (a) or outside the island, on a region also found in *Salmonella Typhimurium* (b). Crosses selecting for the transposon will result in different genotypes depending on the location of the TnGen. If the Tn lies within the *sef* island it will be 100% linked with the *sef* genes in crosses between *Salmonella Enteritidis* and *Salmonella Typhimurium* (a). All recipients must receive the *sef* island. If the Tn lies outside the island (b) recombination can occur between the island and the Tn (i), resulting in inheritance of only the Tn, or distal to the island (ii), resulting in inheritance of the island and the Tn. In this case not all recipients receive the *sef* island. In crosses between *Salmonella Enteritidis* and *Salmonella Enteritidis* normal genetic linkage will be observed.
Salmonella Typhimurium, although there was variable linkage in transductions between Salmonella Enteritidis and Salmonella Enteritidis. This class comprised insertions located in the sef island (Fig. 2a). The Salmonella Enteritidis–Salmonella Enteritidis transductions reflect the expected genetic linkage based upon physical distance between the two markers on the chromosome, but because Salmonella Typhimurium does not contain the sef island, transduction of the Tn5dGen from Salmonella Enteritidis to Salmonella Typhimurium always resulted in inheritance of the sef island. The second class of insertions had a greatly reduced linkage with the sef operon in transductions between Salmonella Enteritidis and Salmonella Typhimurium compared to transductions between Salmonella Enteritidis and Salmonella Enteritidis. This class comprised insertions near the sef island but in chromosomal DNA that is common between Salmonella Enteritidis and Salmonella Typhimurium. Transduction of these markers did not demand inheritance of the sef genes (Fig. 2b).

SefR regulates sef gene expression

The sequence downstream of sefD contained a potential regulator of sef gene expression. This putative gene, which we called sefR, encodes a protein with high homology to members of the AraC-like family of transcriptional activators, with highest homology to the fimbrial regulator subclass of activators (Gallegos et al., 1997) (Fig. 3). Furthermore sefR is located immediately downstream of the fimbrial operon and is convergently transcribed with this operon, a common location for activators of gene expression (Gaastra & Svennerholm, 1996). To determine whether SefR regulates sef gene expression, we tested the effect of SefR on a sefD::MudJ fusion that places the lacZ gene of MudJ under the control of the sefD promoter.

Like other Salmonella, wild-type Salmonella Enteritidis is naturally lac−, thus no β-galactosidase activity was detected in this strain. Low levels of β-galactosidase activity were measured from the sefD::MudJ fusion in an otherwise wild-type cell. However, when sefR expressed from the arabinose-inducible BAD promoter was introduced into the sefD::MudJ strain, β-galactosidase expression was increased even in the absence of arabinose (Fig. 4). When sefR expression was induced by adding 0–2% arabinose to the medium very high levels of β-galactosidase expression were attained (Fig. 4). Together these results indicate that SefR is indeed the activator of sef gene expression.

From the sequences of the sef island, no potential promoter elements between sefA and sefD were

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**Fig. 3.** Alignment of SefR predicted sequence with representative AraC-like transcriptional activators. Salmonella Enteritidis SefR (SEFR_SENT) shows a high degree of similarity to other members of the AraC family of transcriptional activators, particularly those that regulate fimbrial gene expression. Similarity is most conserved over the C-terminal DNA binding region. Identical residues are shown in inverse type. BfpT (BFPT_ECOLI), FasH (FASH_ECOLI), CfaD (CFAD_ECOU) and AggR (AGGR_ECOU) regulate expression of fimbrial operons in E. coli. VirF (VIRF_SHIDY) regulates expression of virulence genes in Shigella spp. (Caron et al., 1989; Edwards & Schifferli, 1997; Jost & Adler, 1993; Nataro et al., 1994; Tobe et al., 1996).
SEF14 fimbriae of Salmonella enterica

Fig. 4. Expression of the sef operon. Low background levels of expression are seen without exogenous sefR. In the absence of arabinose (open bars) pBAD-sefR increased expression of sefD but expression of sefR was unaffected. When 0.2% arabinose was added (solid bars) expression of the sefD::lacZ fusion increased further. When sefA was inactivated by the kanamycin cassette, expression of the lacZ fusion was never detected, confirming the polarity of this insertion. When sefA is inactivated by the non-polar in-frame deletion, expression of the lacZ fusion was unaffected. Solid bars represent cultures induced with 0-2% arabinose, open bars represent uninduced cultures. Data are representative of numerous experiments.

identified that could control expression of sefB, sefC or sefD, suggesting that the sef genes are transcribed as a single operon from a promoter upstream of sefA. The sefD::MudJ fusion was transduced into the various sef mutants to test this hypothesis. β-Galactosidase assays were used to measure sefR-dependent expression of the sef operon in these mutants by comparing expression levels before and after the addition of arabinose to strains containing the AraC-dependent sefR expression plasmid (Fig. 4). Strains which do not have any other mutations in the sef operon except the sefD::MudJ fusion showed approximately sixfold induction of gene expression following the addition of arabinose. Insertion of a polar kanamycin cassette in the first gene in the operon, sefA, abolished sefR-dependent expression of sefD, suggesting that a single transcript encodes the entire operon. In contrast, an in-frame deletion of sefA (ΔsefA) did not reduce sefR induction of sefD gene expression.

Growth-phase regulation of sef gene expression

To investigate the regulation of sef gene expression, β-galactosidase activities were compared at different time points during growth. Expression of both sefR and sefD was optimal during late exponential growth and declined during stationary phase (Fig. 5). Growth-phase-dependent regulation of gene expression appears to occur at the sefR promoter as expression of both sefR and sefD were regulated by growth phase and we have shown that sefR regulates sefD expression. We had previously noted that sef gene expression was higher in a Salmonella Typhimurium LT2 background than in a wild-type Salmonella Enteritidis background. Salmonella Typhimurium has several genetic defects accumulated over years of maintenance in the laboratory. One such defect is a mutation that reduces rpoS expression; thus we reasoned that RpoS may be responsible for both the difference in expression between Salmonella Enteritidis and Salmonella Typhimurium and the growth-phase-dependent regulation of sef gene expression. To investigate whether the difference in expression levels observed between Salmonella Enteritidis and Salmonella Typhimurium could be explained by rpoS effects, an rpoS::Pen mutation was transduced from Salmonella Typhimurium to Salmonella Enteritidis. Because there was no detectable difference in growth rate, β-galactosidase activity was measured after 16 h of growth, when sef expression is normally repressed (Fig. 5). Inactivation of rpoS relieved this repression and the sef genes were expressed at a high level at this time. In contrast, when rpoS was restored on
a plasmid the repression returned (Fig. 6). Together these results suggest that expression of the sef operon is repressed, either directly or indirectly, by the stationary phase sigma factor $\sigma^s$ in *Salmonella* Enteritidis.

**DISCUSSION**

An integrated approach was taken to dissect the sef operon of *Salmonella* Enteritidis. Genomic analysis delimited the island and identified the potential ORFs in this island. Genetic analysis was used to mutate the genes on the sef island and expression studies were used to characterize the regulation of fimbrial gene expression. We showed that the sef operon of *Salmonella* Enteritidis contains four structural genes (sefABC\text{D}) and an adjacent gene (sefR) that encodes an AraC-like regulator of sef gene expression. A single transcript probably encodes the products of sefA through to sefD. SefR, an AraC-like transcriptional activator, regulates transcription of this operon. Expression of the operon is inhibited by the stationary phase sigma factor, $\sigma^s$, encoded by rpoS in response to growth-phase-dependent signals.

Analysis of the available genome sequences from various *Salmonella* serovars revealed a novel large pathogenicity island in all four serovars. From hybridization and PCR studies it was known that the sef operon was located on an island which is absent from many other *Salmonella* but the proximity of phage genomes to this operon had not been appreciated. It remains to be determined whether the phage genomes adjacent to the sef operon are responsible for the transmission of this island between strains. The *Salmonella* Typhimurium genome sequence revealed a large insertion in which only a single ORF had been previously identified. Our analysis suggests that there may be other, as yet uncharacterized, virulence genes in this region.

A region of homology between the virulence plasmid and the chromosome was identified downstream of the sef operon. *Salmonella* Typhi does not contain the virulence plasmid but contains the chromosomal homologue of this region and *Salmonella* Typhimurium contains the region on the virulence plasmid but does not contain the chromosomal homologue. The homology between plasmid and chromosome is potentially only found in a few *Salmonella* serovars, including *Salmonella* Enteritidis, which contain both virulence plasmid and chromosomal homologues. Such regions of homology may allow the plasmid to integrate into the chromosome by homologous recombination.

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**Fig. 5.** Expression of sef genes is regulated by growth phase. The expression of sefD and sefR were measured and were optimal during late exponential growth. Expression of both the sefD::lacZ fusion (solid squares) and the sefR::lacZ fusion (open squares) is maximal during late exponential growth and declines rapidly as the culture enters stationary phase. $A_{550}$ for the wild-type strain is shown on the right axis and represented as open triangles and a dashed line. No difference in growth rate was detected between any of the strains used. No expression was ever observed in LK5, the wild-type strain (solid circles).

**Fig. 6.** Expression of the sef genes is regulated by rpoS. In the absence of rpoS (rpoS::Pen) expression of both sefD::lacZ and sefR::lacZ fusions is increased (note that sefR is not added exogenously). In contrast when rpoS is complemented from a plasmid, expression of sefR is reduced to wild-type levels in an rpoS background.
virulence plasmid is self-transmissible this would result in the formation of an Hfr capable of transferring virulence factors and other genetic elements between serovars (Ahmer et al., 1999). The formation of an Hfr in this way could have profound impact on the emergence of infectious disease and the spread of antibiotic resistance genes.

After this manuscript was first submitted, another group also described the chromosomal region surrounding the sef island (Collighan & Woodward, 2001). There appears to have been a rearrangement around the sef island when their description of the island is compared to Fig. 1. In the strain of Salmonella Enteritidis used in their study, leuX is located downstream of sefD and the sef island is flanked by two insertion elements. They also identify an AraC-like transcriptional activator that they designate SefE. However, when this manuscript was prepared this sequence was not available in the DNA or protein databases and therefore could not be compared to the sequence described here. A third group has also reported the sequence of an AraC-like regulator that is involved in the regulation of the sef operon. This has been deposited in GenBank with the accession number 7330248 (J. A. Botten, I. Kotlarski & R. Morona, unpublished). The two available SefR sequences only differ at two of their 271 residues.

A previous study indicated that sefABC were probably encoded in a single operon (Clouthier et al., 1993). This study also noted that accessory proteins in addition to SefA, SefB and SefC must be required for export of SEF14 fimbriae as no fimbriae were detected on the surface of a smaller clone of the island (Clouthier et al., 1993). The clone that was used in this study was a HindIII subclone from a cosmid. There is a HindIII site in sefD which would produce a clone of the size observed (5.3 kb), suggesting that clones lacked both sefD and sefR, and providing a likely explanation why Sef proteins were not expressed. Another report described a unique 18 kDa fimbria, ascribed to sefD. In this report, antibodies were prepared against the fimbrial protein and used to probe for the distribution of the fimbriae amongst enteric bacteria. Genomic DNA was also probed with a sefD-specific PCR product. Both these techniques suggested sefD, unlike sefABC, had a widespread distribution among enteric bacteria (Clouthier et al., 1994). Several lines of evidence refute this distribution. (i) There is no homology between sefD or its predicted product and the DNA and protein sequences in the E. coli genomic databases even though it was reported that the 18 kDa fimbria can be found in E. coli. (ii) More exhaustive studies using either PCR or Southern hybridization were unable to identify a sefD homologue outside the group D Salmonella (data not shown). (iii) Our studies have shown that a single transcript initiated upstream of sefA and extending through sefD encodes these fimbrial proteins. Moreover, the close coupling of the UGA stop codon for sefC and the AUG initiation codon for sefD indicates that SefC and SefD proteins are co-expressed as expected for components of the same fimbriae. There are no known cases where subunits that form different fimbriae are co-expressed from the same operon, so this close coupling suggests that SefD is part of the SEF14 fimbriae.

Comparison of growth-phase-dependent regulation of gene expression, and the expression of the sef genes in wild-type Salmonella Enteritidis and Salmonella Typhimurium LT2, a natural rpoS mutant, indicated that the sef genes are repressed by σE, the stationary phase sigma factor. These results do not identify whether rpoS affects sef gene expression directly or indirectly and therefore the precise role of rpoS in regulation of sef gene expression, as well as the role of other genetic elements outside of the sef operon remains to be determined.

A previous report showed that the sef genes require a rare arginine tRNA (tRNA-UCU) for expression (Clouthier et al., 1998). It is not known why this tRNA is required. However, genomic analysis of the sef operon showed that the mean G+C content of this region is 35–4 mol%. In comparison, the entire Salmonella Enteritidis genome has a mean G+C content of 52 mol%. The arginine codon recognized by the rare tRNA is AGA. Of the arginine codons in the sef island, 58% are AGA and only 2% are CGC, the preferred Salmonella arginine codon. The limited distribution of the sef island suggested that it is a recent acquisition on the chromosome and this is supported by its unusual codon usage profile. The regulation of the sef operon by tRNA-UCU may simply reflect this different codon usage which presumably betrays some ancestral host with an A/T-rich genome, rather than a specific regulatory mechanism for sensing arginine concentrations.

Although many other fimbrial systems from Salmonella serovars have been identified and characterized, type I fimbriae and the aggregative fimbriae encoded by agfBAC were the only examples for which a transcriptional regulator has been identified (Romling et al., 2000; Tinker & Clegg, 2000, 2001; Tinker et al., 2001). It remains to be seen which elements are required for these systems. Many other fimbrial systems use AraC-like transcriptional activators to regulate gene expression and often the regulator is immediately adjacent to the fimbrial operon, as is found for the sef operon. For example, the 987P fimbria in enterotoxigenic E. coli utilizes a homologous regulator (FasH) to activate gene transcription (Edwards & Schifferli, 1997). The similarity and location of these regulators and the similar organization of the fimbrial operons as a whole [in general they are organized major subunit-chaperone-(optional minor subunits)-regulator] suggests that many fimbrial operons have evolved from some common ancestor.

All of the AraC-like family of transcriptional activators involved in regulation of fimbrial gene expression have similar C-terminal domains. This domain with conserved motifs is the DNA-binding domain. In contrast the N-terminal domains are extremely different. This domain with no conserved motifs is presumably the sensor domain that regulates DNA binding in response...
to environmental cues. This suggests – as practical experience has shown (Edwards & Puente, 1998; Gallegos et al., 1997; Jordi, 1992; Martinez-Laguna et al., 1999) – that each regulator may respond to different environmental stimuli, and hence each fimbria is expressed in different conditions and perhaps in a different location in the host.

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SEF14 fimbriae of Salmonella enterica


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