

# AgfC and AgfE facilitate extracellular thin aggregative fimbriae synthesis in *Salmonella* Enteritidis

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*Salmonella* thin aggregative fimbriae (Tafi; curli) are important in pathogenesis and biofilm formation; however, less is known of their structure and morphogenesis. In the *Salmonella agfBAC* Tafi operon, the transcription and role of *agfC* have been elusive. In this study, *agfBAC* transcripts were detected using a sensitive reverse transcriptase technique. Native AgfC was not detected using polyclonal antibodies generated against purified hexahistidine-tagged AgfC; however, *in trans* expression revealed that AgfC was localized to the periplasm as a mature form. An isogenic  $\Delta agfC$  mutant displayed an abundance of 20 nm fibres, in addition to native Tafi (5–7 nm), and had an increase in cell surface hydrophobicity. Purified 20 nm fibres were depolymerized under exceptionally stringent conditions to release what proved to be AgfA subunits. This revealed that the 20 nm fibres represented a different form of Tafi. The role of AgfC in Tafi assembly was investigated further using an antibody-capture assay of isogenic  $\Delta agf$  mutants. A soluble antibody-accessible form of AgfA was captured in wild-type (*wt*),  $\Delta agfB$  and  $\Delta agfF$  strains, in support of the extracellular nucleation–precipitation pathway of Tafi assembly, but not in  $\Delta agfC$  or  $\Delta agfE$  mutants. This indicates that AgfC and AgfE are important for AgfA extracellular assembly, facilitating the synthesis of Tafi.

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## INTRODUCTION

Fimbriae are cell-surface protein polymers that mediate interactions important for host and environmental persistence, development of biofilms, motility, colonization and invasion of cells, and conjugation (Burrows, 2005; Duguid *et al.*, 1966; Low & Woude, 1996). Four general assembly pathways for different fimbriae have been proposed: chaperone–usher, alternate chaperone, general secretion and extracellular nucleation–precipitation (ENP) (Soto & Hultgren, 1999). In the first three pathways, fibre assembly occurs proximally: in the case of the chaperone-dependent pathways, subunits interact with a chaperone protein that caps interactive surfaces and prevents aggregation in the periplasm, or in the case of the general secretion pathway, subunits pass through a putative cell-envelope-spanning scaffold (Burrows, 2005). The ENP pathway deviates from these systems in that fibre growth occurs extracellularly (Hammar *et al.*, 1996).

Thin aggregative fimbriae (Tafi) are the only fimbriae dependent on the ENP pathway. Tafi were identified in *Salmonella* Enteritidis by Collinson and co-workers, and the operon was termed *agf* (Collinson *et al.*, 1991), whereas in *Escherichia coli*, the homologue discovered was named curli, and the operon termed *csg* (Collinson *et al.*, 1992; Arnqvist *et al.*, 1992). Tafi were later renamed curli using the *csg* nomenclature in *Salmonella* (Romling *et al.*, 1998), but will be referred to by the original *Salmonella* nomenclature of *agf* here. Tafi are known as curli because, in the absence of extracellular polysaccharides (EPSs), their morphology appears curled; however, when expressed with EPS, their morphology appears as a tangled amorphous matrix (White *et al.*, 2003). Together, Tafi and EPS form the extracellular matrix that results in a colony morphotype that appears red, dry and rough (rdar) on Congo red agar (Romling *et al.*, 2000; White *et al.*, 2003), which is highly conserved in most *Salmonella* and *E. coli* strains (Arnqvist *et al.*, 1992; Collinson *et al.*, 1991, 1992; Doran *et al.*, 1993; Gerstel & Romling, 2003; White *et al.*, 2006; Zogaj *et al.*, 2003). Tafi are essential for the formation of the extracellular matrix (White *et al.*, 2006), which is involved in multicellular aggregation (Romling *et al.*, 2000), pellicle formation (Collinson *et al.*, 1993), biofilm formation (Austin *et al.*,

**Abbreviations:** AgfC-his, hexahistidine-tagged AgfC; ENP, extracellular nucleation–precipitation; EPS, extracellular polysaccharide; FA, formic acid; Tafi, thin aggregative fimbriae; TEM, transmission electron microscopy; *wt*, wild-type.

1998; Gerstel & Romling, 2003; Prigent-Combaret *et al.*, 2000; Vidal *et al.*, 1998), environmental persistence (White *et al.*, 2006), and adherence to plant tissues (Barak *et al.*, 2005). Tafi also have pathogenesis-related properties in that they accelerate amyloidosis in mice (Lundmark *et al.*, 2005), bind fibronectin (Arnqvist *et al.*, 1992; Collinson *et al.*, 1993; Olsen *et al.*, 2002), are proinflammatory (Bian *et al.*, 2000, 2001; Persson *et al.*, 2003; Tukul *et al.*, 2005), and enhance adherence and invasion of eukaryotic cells (Dibb-Fuller *et al.*, 1999; Kim & Kim, 2004; La Ragione *et al.*, 2000; Sukupolvi *et al.*, 1997).

The genes involved in Tafi production are organized into two adjacent divergently transcribed operons, *agfBAC* and *agfDEFG* (Collinson *et al.*, 1996). Both operons are required for biosynthesis and assembly (Collinson *et al.*, 1993). *agfA* and *agfB* encode the major and minor fimbrial subunits, respectively (Bian & Normark, 1997; White *et al.*, 2001). *agfD* encodes a transcriptional regulator that positively regulates the expression of Tafi (Romling *et al.*, 1998), cellulose (Romling *et al.*, 2000), O-Ag capsule (Gibson *et al.*, 2006) and serine hydroxymethyltransferase (Chirwa & Herrington, 2003), and negatively regulates factors that inhibit biofilm formation (Prigent-Combaret *et al.*, 2001). The *agfGFEC* gene products are homologous to *csgGFEC* in *E. coli*, where *csgG* encodes an outer-membrane lipoprotein required for fimbrin secretion and stabilization (Chapman *et al.*, 2002; Loferer *et al.*, 1997), and assembles into an oligomeric complex that interacts with the products of *csgEF* (Robinson *et al.*, 2006). CsgE and -F have uncharacterized roles in assembly, but CsgE at least has been called a chaperone (Chapman *et al.*, 2002). The *agfC* (*csgC*) gene has not been previously characterized, and has been referred to as *orfC* due to the lack of evidence for transcription or a distinct phenotype accompanying inactivation (Collinson *et al.*, 1996; Hammar *et al.*, 1995).

In the ENP model of Tafi formation, surface-localized AgfB subunits nucleate the polymerization of AgfA subunits into insoluble surface fibres, which elongate from the free distal end (Hammar *et al.*, 1996). This model relies on the demonstration of intercellular complementation between donor and recipient cells in LPS O-polysaccharide- and cellulose-deficient *E. coli* K-12 (Hammar *et al.*, 1996). In *Salmonella*, intercellular complementation has been demonstrated, but only in LPS O-polysaccharide-deficient mutants (White *et al.*, 2003). Therefore, the significance of intercellular complementation in Tafi-expressing strains with wild-type (*wt*) LPS and other EPSs has been questioned (White *et al.*, 2003).

The purpose of this study was to investigate Tafi assembly, through examination of the minor assembly factors led by the discovery of an active *agfC* gene. The conservation of *agfC* with *agfBA* throughout *Salmonella*, *E. coli* and even in *Shigella*, where the operon has been inactivated (Sakellaris *et al.*, 2000), suggests that *agfC* is functionally important. The predicted amino acid sequence of AgfC has no motifs, domains or homologies in common with any known protein

other than that encoded by *csgC*. In this study, we have shown *agfC* to be co-transcribed with *agfBA*, and that AgfC and AgfE are involved in extracellular Tafi assembly, thereby confirming the ENP model.

## METHODS

**Bacterial strains, growth conditions and culture media.** *S. Enteritidis* 27655-3b (Feutrier *et al.*, 1986) was routinely grown at 37 °C for 24 h on T agar (Collinson *et al.*, 1991), unless stated otherwise. *E. coli* BL21(DE3) (Stratagene), harbouring pET44(c) (Novagen) carrying an inserted sequence of *agfC* fused to the plasmid-encoded C-terminal hexahistidine tag (*agfC-his*), was grown at 37 °C for 18 h with agitation in 1.2% (w/v) tryptone, 2.4% (w/v) bacto-yeast extract and 0.4% (v/v) glycerol broth supplemented with 100 µg ampicillin ml<sup>-1</sup>. *S. Enteritidis* 3b Δ*agfC*, harbouring pARA-A5 (Mayer, 1995) carrying *agfC-his*, was grown at 37 °C for 18 h with agitation in 1% (w/v) tryptone broth (pH 7.2) for cell localization, or T agar for transmission electron microscopy (TEM) experiments. Media were supplemented with 100 µg ampicillin ml<sup>-1</sup> and 0.5% (w/v) L-arabinose. *E. coli* XL-1 Blue (Stratagene) harbouring pBCKS (Stratagene), pHAG (Collinson *et al.*, 1996), pGEM-T Easy (Promega) or pHSG415 (Hashimoto-Gotoh *et al.*, 1981) was grown at 28 or 37 °C for 24 or 48 h with agitation in Luria-Bertani (LB) broth supplemented with 50 µg chloramphenicol ml<sup>-1</sup> or 100 µg ampicillin ml<sup>-1</sup> and 40 mg X-Gal ml<sup>-1</sup>, in addition to 1 mM IPTG, when required.

**RT-PCR.** Cellular RNA from agar-grown cells (OD<sub>600</sub> 1.0) was stabilized with RNA Protect Bacteria Reagent (Qiagen). Cells were incubated with 0.4 mg lysozyme ml<sup>-1</sup> in the presence of Supersase (Invitrogen) and lysed using a QIAshredder (Qiagen). RNA was extracted using an RNA extraction kit (Qiagen), and contaminating DNA was digested with DNase I (Qiagen), according to the method of Wang *et al.* (2002). RT-PCR was carried out using Sensiscript reverse transcriptase (Qiagen), as recommended by the manufacturer, using the primers listed in Table 1.

**Expression and purification of hexahistidine-tagged AgfC (AgfC-his).** *agfC* was PCR-amplified from *S. Enteritidis* 3b chromosomal DNA using primers FagfC and RagfC (Table 1), and fused to a C-terminal hexahistidine tag by cloning into *Hind*III- and *Xho*I-digested pET44(c). *E. coli* BL21(DE3) cells harbouring pET44(c)::*agfC-his* were grown to OD<sub>600</sub> 0.6 before induction with 0.1 mM IPTG at 30 °C for 18 h with agitation (soluble protein), or 1 mM IPTG at 37 °C for 4 h with agitation (insoluble protein). Protein was recovered from cells and purified using 50% (w/v) Ni-nitrilotriacetic acid slurry (Qiagen), as recommended by the manufacturer.

**Production of polyclonal serum against AgfC-his.** Affinity-purified AgfC-his was further SDS gel-purified and used to immunize a New Zealand white rabbit. Subcutaneous and intramuscular injections of 200 µg AgfC-his prepared in Emulsigen adjuvant (MPV Laboratories) were performed three times at 2-week intervals, with a final boost of 100 µg AgfC-his in adjuvant. Three weeks following the final booster injection, serum was collected, and the antibody titre was determined by ELISA using affinity-purified recombinant AgfC-his antigen. The specificity for AgfC was confirmed by immunoblotting and ELISA using affinity-purified recombinant AgfC-his antigen (data not shown).

**Cellular localization.** *S. Enteritidis* 3b Δ*agfC* harbouring pARA::*agfC-his* was grown to OD<sub>600</sub> 0.6, and gene expression was induced with 0.5% L-arabinose at 37 °C for 3 h with agitation. Cells were subjected to osmotic shock (Sweet *et al.*, 1979), and cellular fractions were separated using standard methods (Sambrook &

**Table 1.** Primers used in this study

Primer	Primer sequence (5'–3')	Reference
RT-agfA	TTAATACTGGTTAGCCGTGG	This study
RT-agfC	CTACTGTGCAGAAGCGGCC	This study
agfBF	AGGGTGACAGCATGAAAAAC	This study
agfAR	GTTAGCCGTGGCGTTGTTG	This study
17-A (agfAF)	GCAGAATTCAGCAGTTGTAGTGCAGAAACAGTCGCATAT	White <i>et al.</i> (1999)
agfAR	GTTAGCCGTGGCGTTGTTG	This study
agfCR	GAAGGCGGCCATTGTTGTGA	This study
agfCR	GAAGGCGGCCATTGTTGTGA	This study
agfCF	CTTTATTGCTCCTTGCCCGC	This study
agfCR	GAAGGCGGCCATTGTTGTGA	This study
agfC-C	CATACTTTATTGCTCCGTTAATTAATTAACGTCACATTAACGAACCCTGCGTCTGTCAG	This study
agfC-B	TTCGTTAATGTGACGTTAATTAATTAACGAGCAATAAAGTATGCATAATTTCTCCCG	This study
17-D	AGACGCAAGCTTCGTTAATGTGACCTGAGGGATCACCG	White <i>et al.</i> (1999)
AgfE/F-A	TACTGGAATTCACCGAGGAAAGGATCTC	This study
AgfE-B	AAACGCTATCTGACCTGTTAATTAATTAACAACAGAGGAAGCATTAAATCGCCGACAAAT	This study
AgfE-C	AATGCTTCCTCTGTTGTTAATTAATTAACAGGTGAGATAGCGTTTCATGGCCTTACCGCC	This study
AgfE/F-D	ATAAGAAGCTTATCCACTATTGGCAGGA	This study
AgfF-B	CGTGTAAACATGCAGGTTAATTAATTAACCTCGCGACGGACAGCTCCAGCTCAACGTCAC	This study
AgfF-C	AGCTGTCCGTCGCGAGTTAATTAATTAACCTGCATGTTAACACGCATGACAGCCTCCGG	This study
AgfC2	CCGCAAGCTTCAAAAACACTAGTGCAGAAAGGCGG	This study
FagfC	GGAACATATGCATACTTTATTGCTC	This study
RagfC	CAATCCATCTCGAGCTTGTCGTCATCCTGTGCAGGAAGGCGGCCA	This study

Russell, 2001). Samples were analysed by SDS-PAGE and Western blotting using AgfC-his rabbit serum or isoleucine tRNA synthase control rabbit serum.

**N-terminal sequencing of AgfC-his.** Proteins in periplasmic osmotic shock samples were precipitated in a 10% (w/v) TCA/acetone mixture overnight at 4 °C, washed in ice-cold acetone, centrifuged (16 000 g, 10 min), and allowed to air dry at room temperature for 1 h. The sample was solubilized in 90% (v/v) formic acid (FA), lyophilized, resuspended in SDS-PAGE sample buffer, resolved by SDS-PAGE, transferred to a PVDF membrane (Bio-Rad), and stained with Sypro Ruby protein blot stain (Molecular Probes). Approximately 100 ng AgfC was excised, and eight cycles of Edman degradation were performed.

**Generation of *S. Enteritidis* mutants.** Overlap-extension PCR (Horton *et al.*, 1989) was used to generate deletion constructs for *agfC* (72 bp deletion; primers 17-A, agfC-C, agfC-B and 17-D), *agfE* (72 bp deletion; primers AgfE/F-A, AgfE-B, AgfE-C and AgfE/F-D) and *agfF* (101 bp deletion; primers AgfF/F-A, AgfF-B, AgfF-C and AgfF/F-D) containing six-frame translational stop codons at the beginning of each gene. All primers are listed in Table 1. Gene products were either cloned directly into *EcoRI*- and *HindIII*-digested pHSG415, or subcloned from pGEM-T Easy (Promega). Recombinant plasmids were transformed into *S. Enteritidis* 3b or *S. Enteritidis* 3b  $\Delta$ *bcsA* (White *et al.*, 2003), and used to replace the native alleles in the chromosome (White *et al.*, 2007). PCR was used to screen for deletion strains. Genotypes in final strains were confirmed by sequencing DNA regions of interest that were PCR-amplified from the chromosome. *S. Enteritidis* 3b *fliC*::*Tn10dCm* was generated by random transposition (Gibson *et al.*, 2006).

**Recombinant DNA techniques.** Purified plasmids were electroporated into *S. Enteritidis* 3b or *E. coli* strains using standard techniques (Gene Pulser Electroprotocol; Bio-Rad). Recombinant plasmids were purified using QIAprep spin kits (Qiagen). Restriction enzyme

digestions (New England Biolabs) and ligation reactions (Gibco-BRL) were carried out as recommended by the manufacturers. DNA fragments were purified from agarose gels using Qiaquick Gel Extraction kits (Qiagen). PCRs were carried out using Taq DNA polymerase (Boehringer Mannheim) or Proofstart DNA Polymerase (Qiagen) in buffer supplied by the manufacturer. Thermocycling was carried out in a PTC-100TM Programmable Thermal Controller (MJ Research). All reverse-transcription and PCR primers were purchased from Alpha DNA (www.alphadna.com).

**TEM.** Cells were resuspended in 10 mM Tris/HCl (pH 8.0), placed onto 0.3% (w/v) Formvar-coated 200 mesh copper grids, and negatively stained with 2% uranyl acetate (pH 7) for 15 s. Samples were visualized with a Hitachi H7600 transmission electron microscope under HC-zoom mode at 100 kV.

**Purification of fimbriae from *S. Enteritidis*  $\Delta$ *bcsA* $\Delta$ *agfC*.** Fimbriae from *S. Enteritidis*  $\Delta$ *bcsA* $\Delta$ *agfC* and *S. Enteritidis*  $\Delta$ *bcsA* were purified as described by Collinson *et al.* (1991). Briefly, cells were resuspended in 10 mM Tris/HCl (pH 7.2), vortexed (3 × 1 min) at medium speed, and harvested by centrifugation (6000 g, 10 min). The supernatant samples were lyophilized, resuspended in 750  $\mu$ l SDS-PAGE sample buffer, and boiled for 10 min before electrophoresis (5 h at 150 V). After electrophoresis, SDS-insoluble material that did not enter the stacking gel was recovered from the well, washed in distilled H<sub>2</sub>O (dH<sub>2</sub>O), resuspended in 90% (v/v) FA, and lyophilized. This material was resuspended in 750  $\mu$ l SDS-PAGE sample buffer and electrophoresed a second time. The FA-insoluble material that did not enter the stacking gel was recovered from the well, resuspended in 6 M HCl, microwaved for 5 s at high power (900 W), resuspended in SDS-PAGE sample buffer, and electrophoresed a third time.

**Hydrophobicity assay.** The protocol was carried out according to the method of Rosenberg *et al.* (1980). Cells were washed, resuspended and adjusted to OD<sub>600</sub> 0.6 in 13.6 mM sodium triphosphate

buffer (pH 7.0). Aliquots (4 ml) of cells were overlaid with 1 ml n-octane in acid-washed test tubes. The suspension was mixed vigorously for 1 min by vortexing. The immiscible phases were allowed to separate for 15 min, the aqueous phase was removed, and the  $A_{400}$  was measured. The percentage hydrophobicity was calculated by dividing the decrease in  $A_{400}$  of the aqueous phase by the initial  $A_{400}$ .

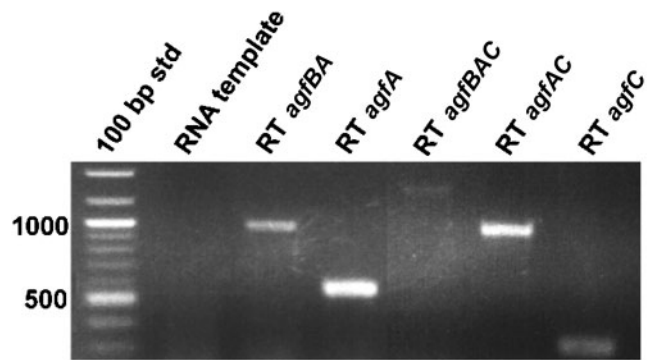
**Antibody capture of Tafi subunits in *S. Enteritidis*  $\Delta bcsA$  strains.** *S. Enteritidis* 3b  $\Delta bcsA$  strains were grown at 37 °C for 20 h with agitation in 1% (w/v) tryptone broth (pH 7.2) supplemented with 30% (v/v) polyclonal rabbit serum specific to AgfA (Collinson *et al.*, 1991) or AgfB (White *et al.*, 2001), with fetal calf serum as a non-specific negative control. For SDS-PAGE and immunoblotting, cells were adjusted to OD<sub>600</sub> 1 in binding buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM Tris/HCl, pH 7.2), centrifuged (6000 g, 10 min), resuspended in 250  $\mu$ l 90% (v/v) FA or dH<sub>2</sub>O, and lyophilized. The supernatant was incubated with 100  $\mu$ l Protein A Sepharose 4 Fast Flow 50% (w/v) slurry (Amersham Biosciences) at 4 °C for 1 h on a rotary shaker. Protein A beads were recovered by centrifugation (16000 g, 10 min), and remaining supernatant proteins were precipitated by addition of acetone (Pohl, 1990). FA-treated samples were loaded directly onto SDS-PAGE columns, whereas all other samples were boiled for 5 min before loading. ELISA was performed according to the method of Engvall & Carlsson (1976) in high-binding, flat-bottomed, polystyrene 96 well plates (Costar). Cells were harvested (6000 g, 10 min), washed twice in 1 ml 100 mM glycine (pH 2.5), washed twice in 1 ml PBS (pH 7.4), adjusted to OD<sub>600</sub> 0.1 in each well, and dried at 90 °C for 4 h. AgfA or AgfB were detected using an AgfA-specific mAb ascites, or AgfB-specific polyclonal serum followed by goat anti-mouse or rabbit IgG-alkaline phosphatase conjugates (Cedarlane Laboratories).

**SDS-PAGE, protein staining and immunoblotting.** Cells were resuspended in 1 ml 10 mM Tris/HCl (pH 8.0) and adjusted to OD<sub>600</sub> 0.1 (for protein staining) or OD<sub>600</sub> 1 (for immunoblotting), and 1 ml aliquots were vortexed (3  $\times$  1 min) to shear off cell-surface material. The cells were harvested by centrifugation (6000 g, 10 min), resuspended in SDS-PAGE sample buffer, and boiled for 10 min before electrophoresis. Proteins in supernatant fractions were precipitated with acetone and resuspended in SDS-PAGE sample buffer. Tafi fimbrial protein samples were prepared as previously described (Collinson *et al.*, (1991)). SDS-PAGE was carried out according to the method of Laemmli (1970), with a 5% (v/v) stacking gel and a 12% (v/v) resolving gel. Proteins separated by SDS-PAGE were either protein-stained with Gelcode (Bio-Rad), or electrophoretically transferred to nitrocellulose using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories), in buffer recommended by the manufacturer. Proteins were detected using immune serum specific to AgfA, AgfB, flagella (*Salmonella* H Antisera; Difco) or AgfC-his, followed by goat anti-rabbit immunoglobulin conjugated to IRDye800 (LI-COR Biosciences). Immunoreactive material was visualized using an Odyssey scanner (LI-COR Biosciences).

## RESULTS

### *agfC* is co-transcribed with *agfBA*

PCR products corresponding to *agfBAC*, *agfAC* and *agfC* were amplified using gene-specific forward primers and an *agfC* reverse primer (Fig. 1). The results indicated that *agfC* was co-transcribed with *agfBA* as a polycistronic *agfBAC* transcript, contrary to the results of previous attempts (Collinson *et al.*, 1996; Hammar *et al.*, 1995).



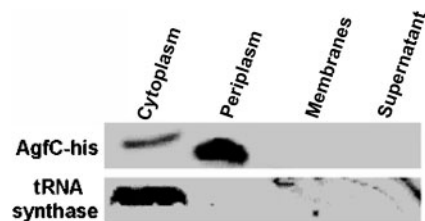
**Fig. 1.** RT-PCR of *agfC*. PCR amplification of *agfBAC* cDNA was performed using primers *agfBF* (*agfBAC*), *agfAF* (*agfAC*) or *agfCF* (*agfC*) with *agfCR*, *agfBF* and *agfAR* (*agfBA*), *agfAF* and *agfAR* (*agfA*). RNA template after DNase digestion was used for negative control PCR reactions. A 100 bp DNA standard (std) (Invitrogen) was loaded, and sizes (in bp) are indicated on the left of the 2% agarose gel.

### Cell localization of AgfC

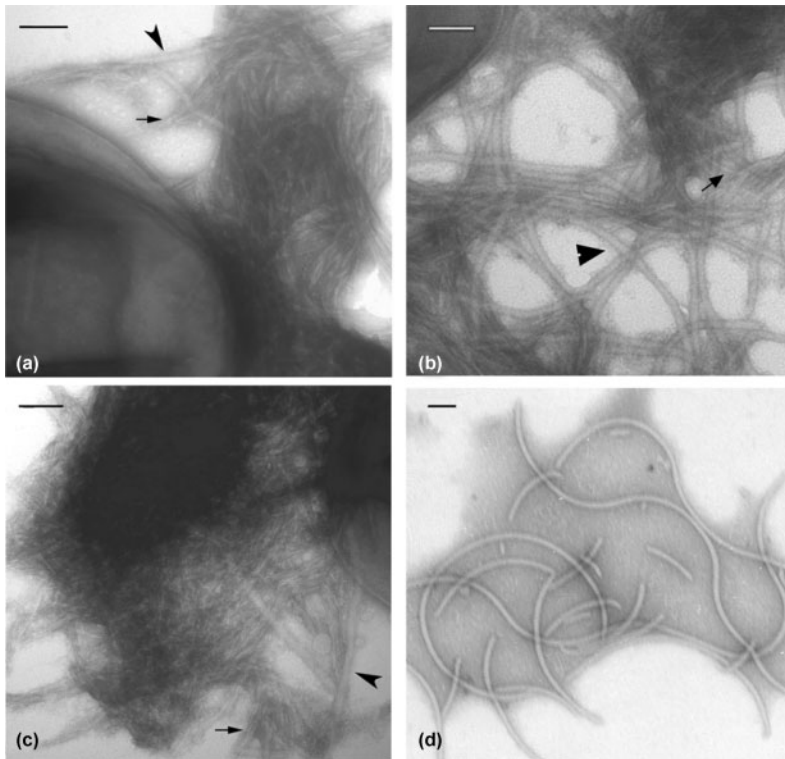
Native AgfC was not detected in *wt* cell fractions using polyclonal immune serum raised against affinity-purified AgfC-his. However, when *agfC-his* was expressed *in trans* in an  $\Delta agfC$  strain, AgfC-his was mostly localized to the periplasm (Fig. 2). N-terminal sequencing of the periplasmic form of AgfC-his revealed that it was cleaved between residues 8 and 9, generating a mature 100 aa protein. This was in contrast to the predicted cleavage site between residues 22 and 23 (SignalP 3.0, [www.cbs.dtu.dk/services/SignalP](http://www.cbs.dtu.dk/services/SignalP)).

### An isogenic $\Delta agfC$ mutant produces aberrant AgfA fimbriae

When *wt* and  $\Delta agfC$  strains were compared by TEM, fibres 20 nm in diameter were abundant on the cell surface of the  $\Delta agfC$  mutant (Fig. 3b), in comparison to the *wt* where fewer 20 nm fibres were observed (Fig. 3a). In contrast to



**Fig. 2.** Immunoblot analysis of AgfC in cellular fractions. *S. Enteritidis* 3b  $\Delta agfC$  with AgfC-his expressed *in trans* was subjected to osmotic shock, and cellular fractions were separated. Proteins were electrophoresed in SDS and analysed by immunoblotting using polyclonal serum generated against AgfC-his or tRNA synthase (control).



**Fig. 3.** TEM micrographs of *S. Enteritidis* 3b cell-surface fibres. Preparations of (a) *wt*, (b)  $\Delta agfC$  mutant, (c)  $\Delta agfC$  mutant with AgfC-his expressed *in trans*, and (d) purified large fibres from the  $\Delta agfC$  mutant were negatively stained with uranyl acetate. Large arrowheads indicate thick ( $\sim 20$  nm diameter) fibres and small arrows indicate thin (5–7 nm) Tafi fibres. Bars, 100 nm. At least 10 images of each strain were generated from at least five independent experiments.

normal Tafi (5–7 nm), these 20 nm fibres were not stained with an AgfA-specific mAb by immunogold-TEM (data not shown). To confirm that the increase in the number of 20 nm fibres was due to the  $\Delta agfC$  mutation, *agfC* was expressed *in trans* in the  $\Delta agfC$  strain; TEM of the complemented strain (Fig. 3c) showed it to be similar to *wt*, with infrequent occurrences of something that resembled the 20 nm fibres on the cell surface. Moreover, 20 nm fibres that had been sheared off cells were enriched in the supernatant from the  $\Delta agfC$  mutant, but not from the *wt* or  $\Delta agfC + agfC$  expressed *in trans* (data not shown).

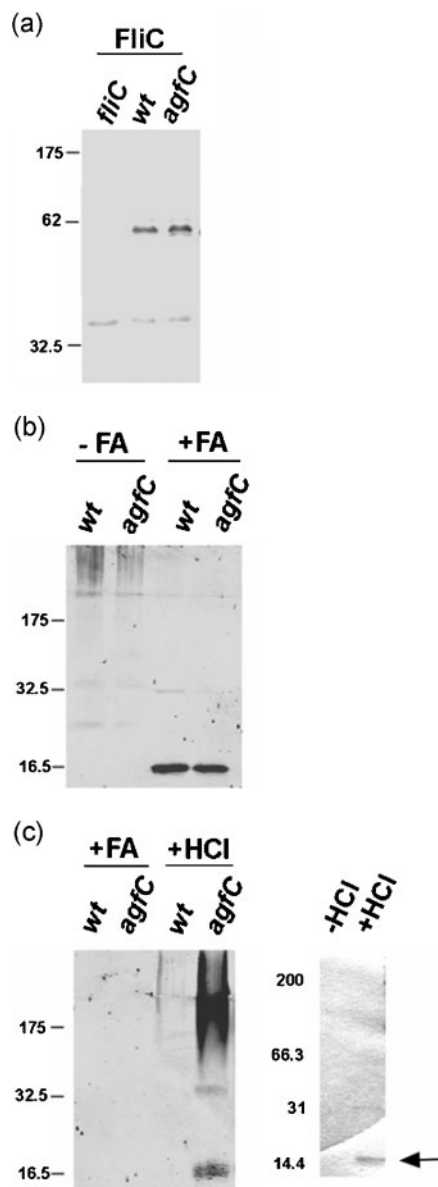
To eliminate the possibility that these large fibres actually represented flagella fragments, supernatants containing sheared surface components from the *wt* and the  $\Delta agfC$  mutant were compared by SDS-PAGE and immunoblotting (Fig. 4a). There were no discernible differences in the quantities of FliC flagellin detected. Additionally, comparison of SDS-PAGE protein profiles from whole-cell lysates of the *wt* and the  $\Delta agfC$  mutant did not reveal any differences between the strains (data not shown). The morphological appearance of these 20 nm fibres, as observed by TEM, was similar in both cellulose<sup>+</sup> ( $\Delta agfC$ ) and cellulose<sup>-</sup> ( $\Delta bcsA\Delta agfC$ ) strains (data not shown), indicating that they did not consist of cellulose-containing complexes.

To determine if the 20 nm fibres were related to Tafi, they were isolated along with Tafi as insoluble material from a  $\Delta agfC$  mutant, according to the previously described Tafi purification method (Collinson *et al.*, 1991). After electrophoresis, the gel-impermeable SDS-insoluble material from  $\Delta agfC$  was recovered from the wells, and found to be highly

enriched with large curved fibres (Fig. 3d). This was in contrast to that of the *wt* strain, which consisted primarily of Tafi fibres. When treated with 90 % FA, AgfA subunits were detected in both strains (Fig. 4b, +FA). However, significant amounts of FA-insoluble material remained in the  $\Delta agfC$  mutant. This material was recovered, washed again with 90 % FA, and solubilized in 6 M HCl by microwave treatment for 5 s. Subsequent SDS-PAGE and immunoblotting revealed a 16.5 kDa protein band and some higher molecular oligomers that were recognized by Tafi polyclonal antiserum (Fig. 4c, +HCl). No corresponding bands were detected in samples from the *wt* strain (Fig. 4c). Analysis of the 16.5 kDa SDS-PAGE band (Fig. 4c, +HCl) by MALDI-TOF MS yielded only peptides that matched the AgfA amino acid sequence (data not shown). Although the AgfA monomers reacted with AgfA-specific polyclonal antisera, they were not strongly recognized using an AgfA mAb that recognizes a conformational epitope (data not shown), suggesting that they were structurally altered. Unlike Tafi fibres, no AgfB subunits were detected in these large fibres by MALDI-TOF MS or Western blotting with AgfB-specific polyclonal serum (data not shown), which readily detects both AgfB and AgfB–AgfA dimers derived from Tafi (White *et al.*, 2003). Consequently, we called these 20 nm fibres aberrant Tafi.

### Changes in *S. Enteritidis* $\Delta agfC$ cell-surface properties

To determine if any surface-related alterations occurred as a consequence of the proliferation of aberrant Tafi, the overall cell hydrophobicity of the  $\Delta agfC$  mutant was compared to



**Fig. 4.** SDS-PAGE and immunoblot analysis comparing cellular proteins in *S. Enteritidis* 3b and the  $\Delta agfC$  mutant. Supernatant samples containing sheared-off surface components were subjected to electrophoresis and analysed by protein staining or immunoblotting. Molecular mass markers (in kDa) are shown to the left of each gel or immunoblot. (a) The relative levels of flagellin were compared by immunoblotting using polyclonal serum generated against FliC (Difco). A *fliC* mutant was included as a negative control. (b) SDS-insoluble material that did not enter the polyacrylamide gel was recovered from the well after electrophoresis, loaded onto a new polyacrylamide gel, and electrophoresed a second time (-FA), or pre-treated with 90% FA (+FA) to depolymerize Tafi prior to electrophoresis. AgfA was detected on corresponding immunoblots using polyclonal serum generated against whole Tafi. (c) FA-insoluble material from the  $\Delta agfC$  mutant or *wt* was recovered from the well after the second electrophoresis (b; +FA), treated with 90% FA again (+FA), or heated in 6 M HCl, electrophoresed a third time, and analysed by immunoblotting using polyclonal serum generated against AgfA (left panel) or protein staining (right panel). The arrow indicates the protein band that was excised and treated with trypsin to generate peptides for analysis by MALDI-TOF MS.

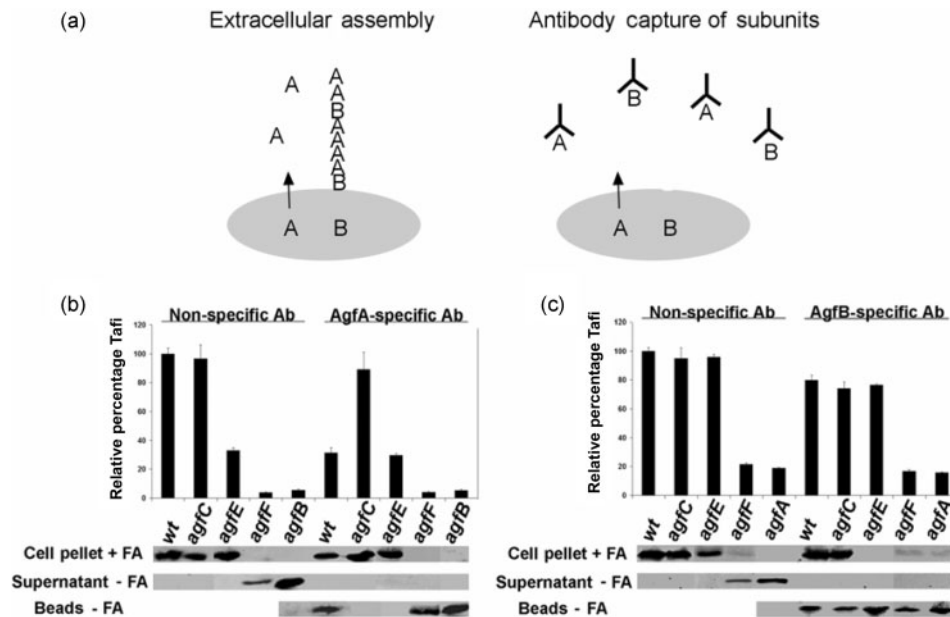
that of *wt*, using the bacterial adherence to hydrocarbons (BATH) test (Rosenberg *et al.*, 1980), which measures the relative hydrophobicity according to partitioning into *n*-octane. The mean  $\pm$  SEM generated from three independent experiments for the  $\Delta agfC$  mutant was  $65.3 \pm 2.3\%$ , whereas the value for the *wt* was  $46.2 \pm 4.3\%$ , representing an approximately 40% increase for the  $\Delta agfC$  mutant.

#### AgfC and AgfE mediate Tafi assembly by the ENP pathway

Since aberrant Tafi were present in the  $\Delta agfC$  mutant, it suggested that the normal assembly process was altered, which implies that AgfC plays a role in correct assembly of native Tafi fibres. We therefore wanted to investigate Tafi assembly in more detail. Strains with isogenic deletions in *agfE* and *agfF*, two proposed Tafi assembly factors (Chapman *et al.*, 2002), were generated to facilitate

comparison with the  $\Delta agfC$  mutant.  $\Delta agfB$  and  $\Delta agfA$  mutants have been described previously (White *et al.*, 2001). To determine whether subunits were accessible to specific antibodies, *wt* and mutant strains were grown in liquid phase in the presence of AgfA- or AgfB-specific immune serum. If the subunits are secreted or surface-accessible as soluble monomers, as proposed in the ENP pathway, we reasoned that specific antibodies would capture the subunits before Tafi polymerization occurred (Fig. 5a). After growth, antibody-subunit complexes were recovered from culture supernatants with Protein A-Sepharose using a pull-down assay, and analysed by immunoblotting. In addition, AgfA or AgfB subunits not captured by antibodies, but incorporated into polymerized Tafi at the cell surface, were measured by ELISA and quantified.

In the *wt* strain grown without serum, AgfA and AgfB were only detected in the cell-associated, polymerized form, and required treatment with FA to enter SDS-PAGE, as expected (Fig. 5, Cell Pellet + FA). In the  $\Delta agfB$  and  $\Delta agfA$  control strains, AgfA and AgfB, respectively, were detected as soluble proteins in the supernatants (Fig. 5, Supernatant - FA). When *wt* cells were grown in the presence of AgfA- or AgfB-specific serum, a proportion of soluble AgfA or AgfB was captured (Fig. 5, Beads - FA). This indicated that during normal Tafi assembly, AgfA and AgfB were in a form accessible to antibodies. The presence of AgfA-specific immune serum caused a 69% reduction in the amount of polymerized Tafi on the *wt* cell surface (Fig. 5b), indicating that the antibodies were competing for polymerization-competent subunits. In contrast, the levels of polymerized Tafi on the cell surface of the  $\Delta agfC$  mutant did not change significantly, and soluble AgfA was not captured with AgfA-specific antibodies. This could be complemented back to the *wt* phenotype with *agfC* expressed *in trans* (data not shown).



**Fig. 5.** Antibody capture of AgfA and AgfB subunits in *S. Enteritidis* 3b strains. (a) Diagram depicting the extracellular polymerization of Tafi subunits, and the capture of Tafi subunits in the presence of specific antibodies preventing Tafi polymerization. (b, c) Cells were grown in the presence of AgfA- or AgfB-specific polyclonal immune serum, respectively. Non-specific immune serum, raised against fetal calf serum, was used as a control. Bar graphs represent the relative amounts of AgfA (b) or AgfB (c) in cell-associated Tafi, as determined by ELISA. Bars represent the average percentage values (compared to *wt* at 100%) from three independent experiments performed in triplicate; error bars represent SD. Insets below the bar graphs show immunoblot analysis of antibody-bound AgfA (b) or AgfB (c) subunits isolated from cell pellet or supernatant samples. Antibody (Ab)-protein complexes were captured using protein A-coated Sepharose beads. The results shown are representative of five independent experiments. FA treatment was used to differentiate between subunits incorporated into insoluble Tafi (+FA), or those present as soluble monomers (-FA).

The  $\Delta agfE$  mutant was similar to the *agfC* mutant, in which basal levels of polymerized Tafi were not altered and soluble AgfA was not captured. This suggested that, in the absence of AgfC and AgfE, AgfA assembly was different and was not proceeding through accessible intermediates. The  $\Delta agfF$  mutant did not produce polymerized Tafi on the cell surface, and soluble AgfA was detected outside of the cell. These results were similar to those for the  $\Delta agfB$  control mutant.

The results of parallel experiments using AgfB-specific antibodies were in contrast to those found using AgfA-specific antibodies: soluble AgfB was captured in all five strains, with a corresponding decrease in the amount of polymerized AgfB detected by ELISA (Fig. 5c). These results indicate that AgfC and AgfE affect the antibody accessibility of AgfA but not of AgfB. These results reveal that AgfC and AgfE facilitate the extracellular assembly of AgfA.

## DISCUSSION

This study implicates AgfC as a Tafi accessory protein, and further defines the role of AgfE. Previously, the *agfC* gene was labelled *orfC*, with no ascribed function. However, we

have demonstrated that *agfC* is co-transcribed as part of the *agfBAC* operon and, along with AgfE, mediates extracellular polymerization of AgfA subunits into Tafi fibres.

*agfC* mRNA was detected by RT-PCR using Sensiscript (Qiagen). Although this technique is not quantitative, we believe that *agfC* is transcribed at low levels, since previous transcription studies using less sensitive techniques did not detect *agfC* (*csgC*) transcripts (Collinson *et al.*, 1996; Hammar *et al.*, 1995). AgfC protein was also produced in minute amounts, as it was not detected in cell extracts. Similarly, other fimbrial systems have minor proteins that are present in minute quantities, making clear definitions hard to assign because of the difficulty of detection (Craig *et al.*, 2003). Although this is artificial, mature AgfC was found primarily in the periplasm when overexpressed *in trans*. Therefore, we hypothesize that the periplasm is the normal cellular compartment for native AgfC.

Deletion of AgfC caused changes on the cell surface of *S. Enteritidis* 3b. The  $\Delta agfC$  mutant cells showed increased cell-surface hydrophobicity and produced 20 nm fibres. We hypothesize that the increase in hydrophobicity is the result of increased production of 20 nm fibres. Although the fibres

are morphologically similar to flagella, the evidence that supports the hypothesis that they are not composed of flagellin subunits but rather of Tafi subunits is: (1) flagella monomer levels were the same in both the *wt* and the  $\Delta agfC$  mutant, despite an increase in the abundance of 20 nm fibres; (2) regular flagella fibres are soluble in SDS buffer and would have disappeared in the first step of the purification procedure; in contrast, the aberrant Tafi fibres were not soluble in SDS buffer; (3) once the fibres were solubilized, AgfA was detected by immunoblotting and MS, whereas FliC was not detected by either technique.

Aberrant Tafi did not depolymerize upon treatment with FA, but required the presence of HCl and heat treatment. After depolymerization, AgfB was not detected, either as protein fragments or immunologically, indicating that the aberrant Tafi were composed solely of AgfA. This is unlike normal Tafi, which are composed of both AgfA and AgfB at a ratio of approximately 20:1 (White *et al.*, 2001). We therefore hypothesize that the AgfA subunits, as present in aberrant Tafi, have adopted an alternative conformation leading to polymerization of thicker filaments, and that this conformation has not conscripted AgfB subunits as in normal Tafi. Formation of this alternative conformation is somehow prevented by AgfC, perhaps by complexing (chaperoning) with pre-assembly AgfA, although we have no direct evidence to show this. Consequently, AgfC biases AgfA assembly toward normal Tafi structure. Fibres morphologically similar to those in the  $\Delta agfC$  mutant have been observed in *E. coli*, in which overexpression of CsgB (AgfB) fused to a maltose-binding protein results in the production of morphologically altered, highly ordered CsgA (AgfA) assemblages that, although uncharacterized, appear curved, loosely aggregated, 10–15 nm in diameter and do not incorporate CsgB (AgfB) (Bian & Normark, 1997). Additionally, others have observed that in the absence of CsgE (AgfE), curli (Tafi) fibres are morphologically altered (Chapman *et al.*, 2002). This indicates that AgfA has the ability to form alternative tertiary, and hence quaternary structures, when normal assembly factors are disturbed.

The role of AgfC in maintaining the conformational integrity of AgfA prompted a further investigation into Tafi assembly factors. The ENP pathway for *E. coli* K-12 curli is based on growth complementation of *E. coli* K-12 CsgA donor (*csgB*<sup>-</sup> or *csgF*<sup>-</sup>) and acceptor (*csgB*<sup>+</sup>) strains, and Congo red detection of polymerizing curli (Bian & Normark, 1997). Since this type of analysis is not feasible with *wt Salmonella* spp. (White *et al.*, 2003), we devised a quantitative analytical test for this system. When *S. Enteritidis* 3b was grown in the presence of AgfA- or AgfB-specific antiserum, soluble subunits were captured and Tafi polymerization was partially inhibited. From these results, it is evident that both AgfA and AgfB monomers are transiently soluble outside the cell before polymerization on the cell surface. This supports the unique ENP pathway for *Salmonella* Tafi, as originally proposed for *E. coli* curli

(Hammar *et al.*, 1996). In contrast, soluble AgfA subunits could not be captured in the  $\Delta agfC$  or  $\Delta agfE$  mutants, even though Tafi polymerization was still occurring. This corroborates previous studies in which a  $\Delta csgE$  ( $\Delta agfE$ ) mutant was unable to donate CsgA (AgfA) subunits when cross-streaked against the CsgB<sup>+</sup> recipient, indicating that, in the absence of CsgE, AgfA is no longer secreted, even though Tafi is still polymerized on the cell surface (Chapman *et al.*, 2002). Together, these results reveal that Tafi assembly can still occur without proceeding through the extracellular-antibody-accessible pathway, demonstrating that extracellular Tafi assembly is not obligatory in these mutants. In the *wt* strain, the ENP pathway accounted for approximately two-thirds of Tafi polymerization when observed this way. One way to interpret these results is that a proportion of Tafi can assemble through an intracellular pathway in the *wt* strain, and in the absence of AgfC or AgfE, the extracellular pathway is lost. Alternatively, in the absence of AgfC or AgfE, the antibody is no longer able to recognize and capture conformationally altered AgfA subunits.

Unlike AgfA, AgfB could be captured by AgfB-specific antibody in the *wt* and the individual deletion strains before polymerization. Therefore, AgfC and AgfE specifically affect AgfA but not AgfB. Thus, the roles of AgfC and AgfE may involve maintaining an assembly-competent AgfA for extracellular polymerization into Tafi. Evidence indicates that the soluble form of AgfA (CsgA) is primarily  $\alpha$ -helical and is not folded into the parallel  $\beta$ -sheet predicted for AgfA in Tafi (Collinson *et al.*, 1999), but it will slowly assemble spontaneously when purified (Chapman *et al.*, 2002). Presumably, the bacterial assembly apparatus (AgfBCEFG) provides the nucleation/assembly platform to prevent premature subunit polymerization while crossing the periplasm, facilitating folding, and accelerating the process. The results here suggest that AgfC and AgfE may be involved in facilitating Tafi assembly by influencing AgfA export and/or conformation, thus facilitating extracellular polymerization. This role is similar to that of chaperone proteins.

This study reports experimental observations of AgfC in Tafi assembly. Experimental observations have been made for CsgE (AgfE) and CsgF (AgfF) (Chapman *et al.*, 2002), but due to the insoluble nature of these fimbriae and their unique assembly pathway, mechanisms involving the Tafi assembly machinery have still not been experimentally defined. AgfC, E and F are all small with no known homologies in the databases. It is at least known that CsgE (AgfE) and CsgF (AgfF) bind CsgG, forming an outer-membrane complex (Robinson *et al.*, 2006).

The detection of the *agfC* transcript in *S. Enteritidis* 3b, and the finding that AgfC and AgfE direct AgfA assembly traffic may be instructive for further studies on Tafi (curli) assembly. These studies may also provide insight into other pathways of aggregative fibre assembly, such as in prion-related diseases.

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