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

D. L. Gibson,1 A. P. White,2 C. M. Rajotte1 and W. W. Kay1

1Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 3P6, Canada
2Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta T2N 4N1, Canada

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 ΔagfC 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 Δagf mutants. A soluble antibody-accessible form of AgfA was captured in wild-type (wt), ΔagfB and ΔagfE strains, in support of the extracellular nucleation–precipitation pathway of Tafi assembly, but not in ΔagfC or ΔagfE mutants. This indicates that AgfC and AgfE are important for AgfA extracellular assembly, facilitating the synthesis of Tafi.

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 curved; 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.
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 (Feurtrie 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⁻¹. S. Enteritidis 3b ΔagfC, 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⁻¹ 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 pHS415 (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⁻¹ or 100 µg ampicillin ml⁻¹ and 40 mg X-Gal ml⁻¹, in addition to 1 mM IPTG, when required.

**RT-PCR.** Cellular RNA from agar-grown cells (OD₆₀₀ 1.0) was stabilized with RNA Protect Bacteria Reagent (Qiagen). Cells were incubated with 0.4 µg lysozyme ml⁻¹ in the presence of Superase (Invitrogen) and lysed using a QIAshredder (Qiagen). RNA was extracted using an RNA extraction kit (Qiagen), and contaminating DNA was digested with DNase I (Fast Digest, Fermentas). First-strand cDNA was synthesized from 0.5 µg RNA using Sensiscript Reverse Transcription Kit (Qiagen). 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 pET44(c). Recombinant protein was recovered from cells and purified using 50 % (w/v) NaCl or 0.5 M (w/v) NaCl supplemented with 100 µg kanamycin ml⁻¹, according to the manufacturer's instructions. The (agfC-his) protein was characterized by SDS-PAGE, N-terminal amino acid sequence analysis and mass spectrometry, and binding to immobilized hexahistidine tag.

**Production of polyclonal serum against AgfC-his.** Affinity-purified AgfC-his was further SDS gell-purified and used to immunize a New Zealand white rabbit. Subcutaneous and intramuscular injections of 200 µg AgfC-his were performed in Emulgen 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 ΔagfC harbouring pARA::agfC-his was grown to OD₆₀₀ 0.6, and gene expression was induced with 0.5 % l-arabinose at 37°C for 1 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’–3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-agfA</td>
<td>TTAATACGTGTTAGCGGTTG</td>
<td>This study</td>
</tr>
<tr>
<td>RT-agfC</td>
<td>CTACGTGTCAGAGGCGGCGC</td>
<td>This study</td>
</tr>
<tr>
<td>agBF</td>
<td>AGGTTGACACATGAAACAC</td>
<td>This study</td>
</tr>
<tr>
<td>agFAR</td>
<td>GTAGCCGTTGCGGTGTTG</td>
<td>This study</td>
</tr>
<tr>
<td>17-A (agAF)</td>
<td>GCAGAATTCGAGGTGTTAGTGCAAGAACATGCGCATAT</td>
<td>White et al. (1999)</td>
</tr>
<tr>
<td>agFAR</td>
<td>GTAGCCGTTGCGGTGTTG</td>
<td>This study</td>
</tr>
<tr>
<td>agCR</td>
<td>GAGGGCCGACTTTGTTGTA</td>
<td>This study</td>
</tr>
<tr>
<td>agCR</td>
<td>GAGGGCCGACTTTGTTGTA</td>
<td>This study</td>
</tr>
<tr>
<td>17-D</td>
<td>AGACGACGACCTTGGTATGTCGAGGATGCGCC</td>
<td>White et al. (1999)</td>
</tr>
<tr>
<td>AgE/F-A</td>
<td>TACTGGAATTCCGAGGAAAGATC</td>
<td>This study</td>
</tr>
<tr>
<td>AgE-B</td>
<td>AAACGCTATCTGAGCGATGTATAATTAACAGAGGACATATATCGGCAATGCAAAT</td>
<td>This study</td>
</tr>
<tr>
<td>AgE-C</td>
<td>AATGCTTCTCCTGTGTTGAATAATTTAACAGGTACATAGGCTGTTTTGCATTGCCCTACGCC</td>
<td>This study</td>
</tr>
<tr>
<td>AgE/F-D</td>
<td>ATAAGAGCTTTACCTACTGGGAGA</td>
<td>This study</td>
</tr>
<tr>
<td>AgF-B</td>
<td>CGTGTIAAACATCGAGGATGTATAATTAACAGAGGACATATATCGGCAATGCAAAT</td>
<td>This study</td>
</tr>
<tr>
<td>AgF-C</td>
<td>AGCTGTCCCTGCAGGATTTAATTGTTAACACCTCAGATGTACCTGAACGTCACGTCGCC</td>
<td>This study</td>
</tr>
<tr>
<td>AgC2</td>
<td>CCAGGAGCCCTAAAACATCTGTGCAAGAGCGG</td>
<td>This study</td>
</tr>
<tr>
<td>FagC</td>
<td>GGAACATGTACATTATTGTGCTC</td>
<td>This study</td>
</tr>
<tr>
<td>RagfC</td>
<td>CAATCCATCTGGAGCTTGTCGTCGCTTACCGTGCAGGAGGCGGCA</td>
<td>This study</td>
</tr>
</tbody>
</table>

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 (16000 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, 17-B, 17-C, and 17-D), agfE (72 bp deletion; primers AgfE/F-A, AgfE/F-B, AgfE/C, and AgfE/F-D), and agfB (101 bp deletion; primers AgfE/F-A, AgfE/F-B, AgfE/C, and AgfE/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 ΔbcsA (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 ΔbcsA ΔagfC 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 Electroporatool: 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 ΔbcsA ΔagfC. Fimbriae from S. Enteritidis ΔbcsA ΔagfC and S. Enteritidis ΔbcsA 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 µl 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 H2O (dH2O), resuspended in 90% (v/v) FA, and lyophilized. This material was resuspended in 750 µl 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.

Hydropobicity assay. The protocol was carried out according to the method of Rosenberg et al. (1980). Cells were washed, resuspended and adjusted to OD600 0.6 in 13.6 mM sodium tripophosphate...
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<sub>600</sub> was measured. The percentage hydrophobicity was calculated by dividing the decrease in A<sub>600</sub> of the aqueous phase by the initial A<sub>600</sub>.

**Antibody capture of Tafi subunits in S. Enteritidis ΔbcsA strains.** S. Enteritidis 3b ΔbcsA strains were grown at 37°C for 20 h with agitation in 1% (v/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/HC1, pH 7.2), centrifuged (6000 g, 10 min), resuspended in 250 µl 90% (v/v) FA or dH<sub>2</sub>O, and lyophilized. The supernatant was incubated with 100 µl Protein A Sepharose 4 Fast Flow 50% (v/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 1 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/HC1 (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 x 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 fimbral 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 Antiserum; Difo) or AgfC-his, followed by goat anti-rabbit immunoglobulin conjugated to IRDye800 (LI-COR Biosciences). Immuno-reactive 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.](image)

**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 ΔagfC 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).

**An isogenic ΔagfC mutant produces aberrant AgfA fimbriae**

When wt and ΔagfC strains were compared by TEM, fibres 20 nm in diameter were abundant on the cell surface of the ΔagfC mutant (Fig. 3b), in comparison to the wt where fewer 20 nm fibres were observed (Fig. 3a). In contrast to...
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 ΔagfC mutation, agfC was expressed in trans in the ΔagfC 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 ΔagfC mutant, but not from the wt or ΔagfC+ 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 ΔagfC 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 ΔagfC 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+ (ΔagfC) and cellulose− (ΔbcsADagfC) 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 ΔagfC mutant, according to the previously described Tafi purification method (Collinson et al., 1991). After electrophoresis, the gel-impermeable SDS-insoluble material from ΔagfC 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 ΔagfC 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 ΔagfC 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 ΔagfC mutant was compared to...
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 ± SEM generated from three independent experiments for the ΔagfC mutant was 65.3 ± 2.3 %, whereas the value for the wt was 46.2 ± 4.3 %, representing an approximately 40 % increase for the ΔagfC mutant.

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

Since aberrant Tafi were present in the ΔagfC 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 ΔagfC mutant. ΔagfB and ΔagfA 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 ΔagfB and ΔagfA control strains, AgfA and AgfB, respectively, were detected as soluble proteins in the supernatants (Fig. 5, Supernatant + 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.
The ΔagfE 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 ΔagfF 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 ΔagfB 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 ΔagfC 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

![Diagram](http://mic.sgmjournals.org)
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 ΔagfC 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 ΔagfC 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^- or csgF^-) and acceptor (csgB^-) 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 ΔagfC or ΔagfE mutants, even though Tafi polymerization was still occurring. This corroborates previous studies in which a ΔcsgE (ΔagfE) mutant was unable to donate CsgA (AgfA) subunits when cross-streaked against the CsgB^- 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 α-helical and is not folded into the parallel β-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 (AgfBCEF) 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.
ACKNOWLEDGEMENTS

We thank Dr E. Ishiguro, University of Victoria, BC, Canada, for the isoleucine tRNA synthetase antibodies and helpful scientific discussions. DNA sequencing was carried out by the Biomedical DNA Sequencing Center (www.uvic.ca/seq), University of Victoria, Victoria, BC, Canada. N-terminal sequencing was performed by the University of British Columbia Nucleic Acid Protein Service Unit, Vancouver, BC, Canada (www.biotech.ubc.ca/services/NAPS), and MS was performed by the University of Victoria – Genome British Columbia Proteomics Centre, Victoria, BC, Canada (www.proteincentre.com). This work was supported through grants to W. W. K. from the National Sciences and Engineering Research Council of Canada (NSERC) and the Canadian Bacterial Disease Network. D.L.G. and A.P.W. were supported by postgraduate and postdoctoral fellowships from NSERC.

REFERENCES


Lundmark, K., Westermarck, G. T., Olsen, A. & Westermarck, P. (2005). Protein fibrils in nature can enhance amyloid protein A


Edited by: D. L. Gally