Molecular Characterisation of the CS5 Pilus Encoding

Region from Enterotoxigenic Escherichia coli O115:H40

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) possess two major virulence factors: the pili or fimbriae that are required for attachment of the bacterium to the intestinal epithelium of the host; and the two enterotoxins, heat-labile and heat-stable, which mediate the characteristic watery diarrhoea observed during ETEC-mediated infection. To date, 21 distinct pilus types have been identified from ETEC strains pathogenic for humans. Of these, detailed molecular studies on pilus biogenesis has been carried out on a select few types. Coli surface-associated antigen 5 (CS5) is an important sub-component of the colonisation factor antigen IV (CFA/IV) expressing ETEC strains, which is present on the surface of 10-20% of all ETEC strains. Despite its obvious importance, very little is known at the molecular level regarding the genes involved in CS5 biogenesis and how the final pilus structure is assembled. This thesis specifically identifies the genes involved in biogenesis and their function, as well as providing a suitable model for assembly of the CS5 pilus.

The region required for biosynthesis of CS5 pilus consisted of six *coli-surface-factor-five* (csf) genes, denoted *csfA-csfF*. The *csfA* gene has been shown by Clark *et al.* (1992) to encode the major subunit. Homology searches showed that *csfC* was similar to a number of outer membrane usher proteins, including CooC of CS1 pili. For the remaining *csf* genes, no other amino acid homologies to other known pilus systems were identified; however, *csfB* was very similar both in sequence and structural terms to *csfF* and likewise *csfD* showed some protein similarity with *csfA*. The region was flanked by a number of distinct insertion sequence (IS) elements, including a complete IS1 and a defective IS30, along with sequences homologous to IS911, IS66 and *orfB* from *Tn21*. Protein expression studies of the *csf* genes showed that each was capable of encoding a protein product in approximate agreement with the predicted molecular masses, with the exception of *CsfC* which migrated faster than predicted. Expression of the *csf* gene cluster without flanking IS sequences from ColE1-based plasmids in *E. coli* K-12 produced large amounts of peritrichous CS5 pili, which served as a
suitable system for mutagenesis studies and functional analysis of each of the newly identified csf genes.

Two minor pilins were identified in this thesis, which were absolutely required for pilus initiation and termination. Firstly, examination of pilus biogenesis in a csfD deletion strain showed that CsfD was required for pilus initiation and did not function as a specific chaperone for the major subunit. Western analysis on purified CS5 pili showed that CsfD was an integral minor subunit, constituting between 1.2-1.8% of the pilus. The association of CsfD within the pili, composed primarily of CsfA, was a stable interaction, and could only be liberated upon boiling of the pili. Direct localisation of CsfD within the pili by immunogold electron microscopy was subjective due to bundling of the pili. Ultrastructure analysis of purified CS5 resolved the pilus morphology to be a flexible 2 nm fibrillae, with CsfD predicted to localise at intervals along the shaft and at the distal tip. The association of CsfE and termination of pilus assembly was shown directly by constructing a deletion in the csfE gene, which resulted in extended pilus morphologies. Complementation with a wild type copy of the csfE gene on a number of different expression plasmids was able to shorten the mean length of the pili, although CsfE was not rate-limiting for termination of pilus assembly. Fractionation studies showed that CsfE localised in the outer membranes and was not detected in crude or purified CS5 pili.

The csfC gene was shown to act as an outer membrane usher protein for CS5 biogenesis, since CsfC was localised in the outer membranes by fractionation experiments and a deletion in the csfC gene resulted in the complete absence of pilin subunits in the supernatant of this strain. The specificity of the CsfC usher for the pilin subunits was demonstrated by the inability of homologous ushers from the CS1 and CFA/I pilus systems to translocate and indeed assemble CS5 subunits to form cell surface pili.

Two chaperone-like proteins were identified which possessed specificity for either the major subunit CsfA or the minor subunits CsfD and CsfE. CsfB was shown to specifically stabilise the major subunit CsfA, since a deletion in the csfB gene resulted in the complete...
absence of CsfA in periplasmic fractions prepared from the strains. Complementation experiments with a wild type copy of the csfB gene was able to restore the accumulation of CsfA in the periplasm and cell surface CS5 pilus expression, as judged by immunogold electron microscopy. Fractionation studies also indicated that CsfB was located in the periplasm of the bacteria and was not associated with the final pilus structure.

An in-frame deletion in the csfF gene was shown to result in an extended pilus morphology by immunogold electron microscopy, and such pilis contained no detectable levels of CsfD by western immunoblot analysis. CsfF was shown to stabilise CsfD in the periplasm of the bacteria by constructing a deletion in both csfF and csfB genes and re-introducing a wild type copy of the csfF gene back into the system. CsfF was shown to be rate-limiting in terminating pilus assembly, since modulating CsFF expression resulted in significantly shorter pilis versus the wild type strain. Therefore, CsfF is predicted to deliver CsfE to the outer membrane to terminate assembly, and the rate of incorporation of CsfE is dependent on CsFF levels in the periplasm.

A model for CS5 pilus biosynthesis has been proposed in this thesis. Initiation of pilus biogenesis occurs when a CsfD-CsfE complex is delivered to the CsfC outer membrane usher protein, which results in the translocation of the CsfD minor pilin subunit across the outer membrane. This event marks a conformational change in CsfC, thereby allowing CsfA-CsfB complexes to be delivered and CsfA major subunits translocated across the outer membrane and polymerised into the growing pilus. Further CsfD-CsfE complexes are also incorporated into the pilus at a rate determined by the stoichiometric ratio of CsfA and CsfD in the periplasm. Termination of pilus assembly occurs when a CsfE-CsfF complex is delivered to CsfC, resulting in the incorporation of CsfE, which prevents further polymerisation of the pilus.
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