The Level of Expression of the Minor Pilin Subunit, CooD, Determines the Number of CS1 Pili Assembled on the Cell Surface of Escherichia coli

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CooD, the minor subunit of CS1 pili of enterotoxigenic Escherichia coli, is essential for the assembly of stable, functional pili. We previously proposed that CooD is a rate-limiting initiator of CS1 pilus assembly and predicted that the level of CooD expression should therefore determine the number of CS1 pili assembled on the cell surface. In this study, we confirm that CooD is required for the initiation of pilus assembly rather than for the stabilization of pili after they are assembled by demonstrating that specific modulation of cooD expression also modulates the number of CS1 pili on bacterial cells.

CS1 pili represent a family of adhesins associated with enterotoxigenic Escherichia coli (ETEC) that is pathogenic for humans and associated with Burkholderia cepacia, a pathogen associated with cystic fibrosis (23). Pili belonging to this family, including CS1, CFA/I, CS2, and CS14, mediate the binding of ETEC to human enterocytes in vitro (4, 11, 28) and are therefore thought to be necessary for colonization of the host intestine. This has been confirmed for CFA/I pili, which have been shown to be necessary for the maintenance of ETEC in the intestines of human volunteers (6) and for the CS1-related Cbl type II pili of B. cepacia which have been implicated in binding to respiratory mucins (20, 21).

The CS1 family is one of three major classes of pili associated with gram-negative bacterial pathogens. It is distinguished from the other two major families, the type IV and Pap (pseudomonas-associated pilus)-related pili families, primarily by a lack of sequence similarity with any of the proteins involved in pilus assembly (23). Both the type IV pilus systems, which require up to 14 genes for the assembly of pili (16, 26, 27), and many Pap-related pilus systems, which may require up to 9 piliassembly genes (9), are relatively complex. In contrast, for assembly, CS1 pil are required only the four cotranscribed genes cooB, -A, -C, and -D (7, 15, 17, 25).

CS1 pili are composed almost entirely of the major subunit CooA, and contain on their tips CooD, which is estimated to contribute only one subunit per pili (22). The assembly of CooA and CooD subunits into pili depends on the presence of two other proteins encoded by the coo operon: CooC, a large outer membrane protein that is probably involved in the transport of pili across the outer membrane (22, 29); and CooB, a periplasmic chaperone-like protein that forms intermolecular complexes with each of the pili in the periplasm and with CooC in the outer membrane and stabilizes these proteins (22, 29). CooD and CooA-related pili share important morphogenetic features, including the transport of pili via the periplasm, a requirement for a periplasmic chaperone, and a large outer membrane assembly protein. Since the proteins of CS1 and Pap-related pili are unrelated, it seems likely that these similarities result from convergent evolution (22, 29).

Although CooD is a minor pilin, it is also essential for the assembly of functional pili (7). If CS1 pil are assembled like type I pili by incorporation of subunits at the base of the pili (14), the location of CooD at the pilus tip (22) suggests that it is probably the first subunit incorporated into the pili. The absolute requirement for CooD in pilus assembly, the location of CooD at the pilus tip, and the very low level of CooD expression in wild-type cells (22) suggest that CooD may be a rate-limiting initiator for the assembly of CooA subunits into pili. In this model, CooD is essential for the initiation of pilus assembly and so the level of CooD expression should determine the number of pili on the cell surface (22).

Because pili are not detectable in the absence of CooD on the tip (7), the alternative model is that CooD is added last but is needed to stabilize the pilus structure. This model predicts that in the absence of CooD, CooA is secreted to the cell exterior but does not remain polymerized in a pilus structure. To test these models, we have investigated whether CooA is secreted to the cell exterior in the absence of CooD and whether modulating the expression of CooD influences the number of pili assembled on the cell surface.

CooD is necessary for the extracellular transport of CooA. To investigate the extracellular secretion of CooA in a cooD mutant, the coo genes were expressed in the E. coli ara deletion mutant LMG194 (8) from plasmid pEU1290 which carries cooB, cooA, and cooC expressed under the pTrc99A promoter regulated by isopropyl-β-D-thiogalactopyranoside (IPTG) (1). For the construction of pEU1290, an insert carrying cooB, cooA, and cooC was amplified by PCR from the template plasmid pEU494 (7), using Pfu DNA polymerase (Stratagene) and the oligonucleotide primers BACUP (5'AAATGCTTTTTCATTCAGTATCCTGATTG3') and BACDOWN (5'AAATGCTTTTTCATTCAGTATCCTGATTG3'). The 4.1-kb insert was digested with Hinfl and cloned into the corresponding restriction sites in pTrc99A to place cooB, -A, and -C under the control of the PTrc promoter.

Bacteria were grown in Luria-Bertani (LB) medium (24) with aeration at 37°C in the presence of ampicillin (100 μg/ml) and 0.1 mM IPTG to induce expression of cooB, -A, and -C, and extracts were assayed by immunoblotting with anti-CooA antisera. Although no CooA was found in extracts of cells plus supernatant of LMG194, as expected, CooA was readily

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detectable (data not shown) in extracts of the LMG194/pEU1290 culture containing both bacteria and supernatant. A comparison between the dilutions of this extract and an extract from undiluted supernatant of this strain from which the bacterial cells had been removed showed that the supernatant contained between 1 and 10% of the total CooA present (data not shown). Immunoblots showed that the fraction of CooA in the culture supernatant was not greater than that of MalE, a periplasmically located protein that would be present in the supernatant only if the cells lysed (data not shown).

The stability of CooA subunits was tested with monomeric CooA isolated from purified CS1 pilus by boiling the pili for 20 min in sterile distilled water, as previously described for CFA/I pilins (2). These subunits were added to a culture of LMG194 and incubated at 37°C for 2 h. Immunoblots showed that the concentration of subunits did not change (data not shown), so if CooA had been secreted from the cooBAC strain, it should have been detectable. We conclude that CooA does not appear to be secreted to the cell exterior in the absence of CooD.

To investigate whether the level of cooD expression influences the number of CS1 pili assembled on the cell surface, we used a two-plasmid system which allowed us to specifically modulate the expression of cooD while keeping the expression of cooB, -A, and -C constant. Plasmid pEU1290 carries cooB, -A, and -C under control of the IPTG-inducible P_{trc} promoter, and plasmid pEU1206 carries cooD under the transcriptional control of the arabinose-inducible ParaBAD promoter (8). To create pEU1206, the HindIII-BglII cooD fragment from pEU493 (23) was blunt ended, passed through an intermediate vector to provide appropriate flanking restriction sites, and cloned into pBAD30 (8) to which the 1.9-kb spectinomycin resistance cassette of pHP45 (18) had been added at the FspI site.

For full repression of the ParaBAD promoter on multicopy plasmids, glucose-mediated catabolite repression is usually needed (8). To confirm that glucose represses and arabinose induces cooD expression in E. coli LMG194/pEU1290/pEU1206, immunoblots of whole-cell extracts were probed with anti-CooD. As expected, no CooD was detectable in the negative-control strain LMG194/pEU1290 (cooBAC') (Fig. 1, lane 1). When grown in the presence of glucose, LMG194/pEU1290/pEU1206 (cooBAC' cooD') did not produce enough CooD to be detected either (Fig. 1, lane 2). However, in the presence of arabinose, CooD was easily detected in this strain as a 38-kDa protein and a 25-kDa truncated product (Fig. 1, lanes 3 to 5) which has been described previously (22, 29). Using immunoblots, we have also confirmed that neither glucose nor arabinose affects the expression of CooA from LMG194/pEU1290 (cooBAC') (data not shown).

Effect on CS1 piliation of modulating cooD expression. To examine the effect of cooD expression on CS1 piliation, cultures of E. coli LMG194/pEU1290/pEU1206 were grown in LB broth containing ampicillin (100 μg/ml) and spectinomycin (100 μg/ml) in the presence of 0.1 mM IPTG to derepress cooB, -A, and -C. The number of pili on bacteria from cultures in which cooD was repressed by growth in glucose (0.2% [wt/vol]) or induced by addition of arabinose (0.002, 0.01, or 0.05% [wt/vol]) was determined by electron microscopy of broth-grown cells collected by centrifugation (as described previously [22]). In the negative-control strain LMG194/pEU1290, no pili were detected, as expected (data not shown). When glucose
was used to repress cooD expression, some pili (average of 28 pili/cell) were detectable on the bacterial surface (Fig. 2 and Table 1). Since CooD is required for the production of pili, it appears that there is some expression of cooD even in the presence of glucose. However, when cooD expression was induced with arabinose, the number of CS1 pili per cell increased dramatically, with most cells producing >150 pili per cell (Fig. 2 and Table 1). Therefore, the level of cooD expression determines the number of CS1 pili assembled on the cell surface.

**Conclusion.** From the work presented here, we conclude that CooD is essential for the extracellular transport of CooA, the major pilus subunit. As expected, the level of CooD expression controls the number of CS1 pili assembled. This is consistent with the model we previously described (22, 29) in which CooD is the rate-limiting initiator of pilus assembly. In this model, the major pilus subunit CooA cannot be transported through the outer membrane and polymerized into pili until CooD has assembled the pilus structure. Because it is required to initiate pilus assembly and be transported through the outer membrane to form the pilus structure, CooD is the rate-limiting initiator of pilus assembly. In this model, the major pilus subunit, and that the level of CooD expression was in-