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Permissible peptide insertions surrounding the signal peptide-mature protein junction of the ClpG prepilin: CS31A fimbriae of \textit{Escherichia coli} as carriers of foreign sequences

(Recombinant DNA; random mutagenesis; signal sequence; genetic fusions; hybrid protein secretion; chaperone; transmissible gastroenteritis virus; epitope; antigen display; pilin)

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SUMMARY

The clpG gene, expressing the \textit{Escherichia coli} major CS31A fimbrial subunit ClpG, was subjected to random mutagenesis by insertion of an EcoRI linker and a kanamycin-resistance (KmR) cassette into the multiple newly generated EcoRI sites. The KmR gene was then excised by PstI, which left a 48-bp linker representing the heterologous sequence. The same procedure was followed to introduce a synthetic oligodeoxyribonucleotide (oligo) corresponding to epitope C from the spike protein S from the porcine transmissible gastroenteritis coronavirus (TGEV). Nine insertion/deletion mutants (indels) that contained long foreign peptides variously located around the ClpG signal peptide (SP) processing site were characterized. A striking feature of this study is the variety of amino acid (aa) insertions in the ClpG prepilin that have little or no effect on CS31A fimbria biogenesis. These 'permissive' sites tolerate inserts of 18 or 19 aa and accept sequences of different natures in view of their aa composition, charge and hydrophobicity. The results obtained here are also interesting in light of the high level of aa sequence conservation seen in the SP and N-terminal domains of the ClpG-related subunits. The structure-function relationship of the ClpG SP is discussed. The TGEV-C epitope fused to the N-terminal end of the mature ClpG protein was cell-surface exposed, as observed on immuno-electron microscopy. Therefore, the CS31A fimbria seems to be a potent tool for the presentation of foreign antigenic determinants or the production of heterologous polypeptides in \textit{E. coli}.

INTRODUCTION

Polymer CS31A is a plasmid-encoded fibrillar protein associated with animal and human pathogenic isolates of \textit{Escherichia coli} (Contrepois et al., 1989; Cherifi et al., 1990). The clp gene cluster, which contains seven structural genes (clpC, clpD, clpE, clpF, clpG, clpH and clpI), encodes all the secretory protein products required for
CS31A biogenesis (Martin et al., 1991). They include the major ClpG monomer and several accessory proteins in involved in the stabilization, transport and assembly of ClpG. Extensive nt sequence homology throughout the accessory protein-encoding genes of CS31A. K88 and F41 operons has been demonstrated by Southern hybridization analysis (Casey et al., 1990; Martin et al., 1991; Korth et al., 1991). The accessory systems mediating expression of these operons may be functionally interchangeable, indicating that CS31A, K88 and F41 fimbriae are members of a closely related family (Korth et al., 1992). The clpG gene codes for the ClpG precursor containing a typical Gram- bacterial 21-XI SP, whose fimbriae arc members of a closely related family (80%) SP was observed for ClpG, FaeG and F41 prepilin, respectively (Girardeau et al., 1991). By analogy with the known K88 fimbriae. the extensive modifications engineered in ClpG SP, the signal sequence-surrounding region (region I). The central part of ClpG (region III), and the C-terminal end of ClpG (region IV). Only region IV of pilin seemed to be both permissive, variable, hydrophilic and non-secondary structured.

Because no studies have been made to characterize the effects of signal sequence mutations on the production of E. coli fimbriae, the only heterologous insertions accurately located by DNA sequencing were those confined to the signal sequence-surrounding region (region I). The properties of these mutants, ClpG 9/10.1, ClpG 15/29.1, ClpG 16/21.1 and ClpG 21/22.1, are described in detail in section d.

(c) Insertion of the TGEV-C epitope and immunoreactivity of the hybrid ClpG proteins

Synthetic oligo encoding the C epitope processing site (aa 361-372) from surface protein S of porcine coronavirus was flanked by EcoRI cohesive ends (Fig. 4A). Plasmid DNAs isolated from mutants ClpG 9/10.1, ClpG 15/29.1, ClpG 16/21.1 and ClpG 21/22.1 (section b), were restricted by EcoRI, religated in the presence of an excess of the epitope C-oligo insert, and then complemented by pDSPH524 in E. coli DH5a. Colonies with a TGEV-C epitope insertion were screened and analysed by in situ immunoblotting for CS31A fimbria production and C epitope antigenicity. All of the colonies reacted with anti-ClpG pAb but only 25% with the neutralizing site-C-epitope insertion were screened and analyzed by PAGE both

RESULTS AND DISCUSSION

(a) Construction of a trans-complementation system

To perform the DNA manipulations in the clpG subunit gene without affecting the rest of the operon carrying the helper-genes, we subcloned clpG and clp helper-genes into two separate compatible plasmids (Fig. 1). One of the two resulting recombinant plasmids, pDSPH524 contained all the accessory genes, and the other, pDEV41155, carried only clpG. In complementation experiments, pDSPH524 and pDEV41155 were co-selected on the basis of their Cm and Ap resistance, respectively. The clp helper-genes encode trans-acting proteins allowing the stable expression of clpG, and therefore capable of mediating the biogenesis of CS31A fimbriae. No production of CS31A occurs in E. coli containing only either pDSPH524 or pDEV41155.
Fig. 1. Construction of the CS31A complementation system. The genetic and physical maps of the hybrid plasmids pEH524, pDSPH524 and pDEV41155 are shown. Plasmids pEH524 and pDSPH524 are low-copy-number plasmids containing a pSC101 replicon, whereas pDEV41155 is a high-copy-number plasmid carrying a pColE1 replicon. Plasmid pEH524 was previously constructed by cloning a 8.5-kb EcoRI-HindIII DNA fragment containing the CS31A operon with its own promoters between the EcoRI and HindIII sites of the pHSG575 vector (Martin et al., 1991). To construct a plasmid containing only the clp helper genes, clpC was deleted from the CS31A operon by SphI restriction of pEH524 to give pDSPH524. c/pG was isolated from pEH524 as a 1.12-kb PstI-Hpal fragment and inserted into PstI+HincII-digested pUC19. A 0.91-kb EcoRV-XbaI fragment from this new construct was inserted into EcoRV+Xh1I-digested pBSK+ vector to give pDEV41155. The EcoRV-Xbal fragment that was placed downstream from a strong vector promoter encoded the ClpG precursor protein and contained the three stop codons terminating the open reading frame of clpC. Boxes indicate sizes and locations of the various structural clp genes. The names of the genes are given in the boxes and the sizes (in kDa) for the mature proteins are beneath the boxes. The black area on the left side of the clpG box represents the part of ClpG coding for the SP. The major fimbrial subunit (pinn) of CS31A fimbria is indicated by an asterisk (after the kDa value). The arrows indicate the direction of transcription: P, promoter of the clp gene cluster; PG, weak promoter of clpG; Plac, promoter of the lactose operon; T, terminator of transcription in the clp gene cluster; TG, terminator of transcription of the clpG gene. Ap, Apal; El, EcoRI; EV, EcoRV; Hc, HincII; Hd, HindIII; Hp, Hpal; Ps, PstI; Sa, Sulf; Sc, SacI; Sp, SphI; Xb, Xbal; Xh, XhoI; Δ, deletion; pBSK+, pBluescript SK(+) (Stratagene, La Jolla, CA, USA).

under SDS-denaturing and native conditions. Under denaturing conditions, mutant ClpG 21/22.3C produced a protein reacting with both Ab and exhibited a slower migration than wt ClpG (Fig. 4B). The M, of the monomeric form of the hybrid corresponded to the 257 aa of ClpG plus the 18-aa extension, which includes 12 aa corresponding to the TGEV-C epitope. The ability of hybrid fimbriae to oligomerize was evidenced under non-denaturing conditions with specific anti-ClpG pAb. A difference in migration between the multimeric forms of the native ClpG and native hybrid ClpG::TGEV-C epitope proteins was observed (Fig. 4B), presumably due to the negative net charge (−3) of the oligo insert. Immuno-EM confirmed the presence of the TGEV-C epitope in hybrid fimbriae surrounding the ClpG 21/22.3C cells (Fig. 4C). The method involved the epitope recognition by the 3b.5 mAb and its indirect labeling with goat anti-mouse immunoglobulin G (IgG) conjugated with gold particles. Taken together, these findings demonstrate that epitope C is properly exposed on the monomeric and oligomeric forms of the native hybrid protein at the E. coli cell-surface.

Of the remaining 98% of clones that reacted only with anti-ClpG pAb, some were analysed by plasmid-DNA sequencing. In all cases, the TGEV-C epitope-encoding oligo was inserted in-phase in the incorrect orientation and gave mutants ClpG 9/10.2, ClpG 15/29.2, ClpG 16/21.2 and ClpG 21/22.2. This may have been because insufficient clones were analysed to exhaust all the possibilities.

(d) Characterization of preClpG mutants

The examination of the aa sequence immediately upstream from the experimentally determined processing site (Girardeau et al., 1988; Fig. 5) shows that the 21-aa SP of the preClpG has a perfect similarity to typical Gram− bacterial, signal peptidase-I-processed SP sequences normally present at the N-termini of proteins destined to be translocated through the cytoplasmic membrane by the SecA-mediated pathway (Inouye and
Fig. 2. Mutagenesis by random insertion into clpG gene. (A) To create random polynucleotide insertions, pDEV41155 was cleaved with DnaI (BRL) in the presence of Mn$^{2+}$, as described by Smith and Crouse (1989), to favour the production of single-cut plasmids. The ends were filled-in with T4 DNA polymerase and PolH plus four dNTPs (Pharmacia). Full-length blunt-ended fragments of the appropriate size were then ligated overnight at 4°C with an excess of unphosphorylated EcoRI linkers as described by Lathe et al. (1984). To maintain the correct reading frame, five double-stranded EcoRI linkers were used in five simultaneous independent ligation-transformation experiments: d(GGAATTCC), d(CGCGAATTCCG), d(CCCGAATTCCGG), d(CCGGAATTCCG), and d(CCCGAATTCCGG). After ligation, the T4 DNA ligase was inactivated at 65°C for 15 min and the ligation mixture was used to transform E. coli DH5α (BRL). Transformants were grown overnight in liquid medium instead of the usual plating on solid medium. Resulting plasmids were isolated from stationary phase cultures by alkaline lysis (Birnboim and Doly, 1979) and ligated overnight at 4°C with the KmR cassette derived from plasmid pUC4K (Vieira and Messing, 1982) as described by Philpott and Zimmerman (1983). E. coli DH5α transformants were selected on LB plates containing Ap and Km, at concentrations of 100 and 50 μg/ml, respectively, and Ap$^{R}$Km$^{R}$ clones harboring pDEV41155 with Km$^{R}$ cassette were screened. To select Km$^{R}$ cassette insertions located only in the ClpG gene, a pool of plasmid DNAs was prepared from 100 clones Ap$^{R}$Km$^{R}$ and restricted with both Apal and SacI. Apal/SacI fragments of 2.25 kb containing the mutated clpG gene were cloned into Apal-SacI sites of the phagemid pBSK+ vector (Stratagene). After transformation a pool of plasmid DNAs was prepared from 100 Ap$^{R}$Km$^{R}$ clones. Heterologous sequence insertions of desired size were created in clpG by PstI excision of the Km$^{R}$ cassette. Linearized plasmids devoid of the Km$^{R}$ cartridge were purified, religated and transferred into E. coli DH5α [pDPS85241]. 12.5% of the Ap$^{R}$Cm$^{R}$Km$^{R}$ clones produced cell surface CS31A fimbriae as determined by colony immunoblotting (Guesdon et al., 1985) with a specific anti-ClpG pAb (Girard et al., 1988). These clones represent insertion sites that were permissive for the introduction of 16 extra aa in the ClpG protein. The distribution and the location of these permissive insertions were approximately determined by Sall restriction analysis of a pool of plasmid DNAs from 100 immunoreacting clones. (B) The nt and aa sequences of MCS inserts in the three reading frames, depending upon the type of EcoRI linker previously used.
Holegona, 1980). The ClpG SP is considerably different from the SP of type IV pilins or homologues that are transported through a specialized Sec-independent export machinery and processed by a cognate prepilin peptidase (see, for a review, Hobbs and Mattick, 1993).

We located by DNA sequencing nine mutations generated by random oligo-insertion mutagenesis which specifically altered regions surrounding the signal sequence of clpG without drastically affecting the CS31A fimbria biogenesis on the basis of colony- and Western immunoblotting (data not shown) or immuno-EM (only for ClpG 21/22.3C mutant; Fig. 4C). In all cases, depending on the reading frame and surrounding nt, 18 or 19 aa were inserted in-phase into the different sites at positions −13/−12, −7/+8, −6/−1 and −1/+1 (Fig. 5). In some cases the random insertions were accompanied by deletions corresponding to the loss of 4 aa (mutants ClpG 16/21.1 and ClpG 16/21.2) or 13 aa (mutants ClpG 15/29.1 and ClpG 15/29.2; Fig. 5 and Table I). The final modifications are insertions with or without deletions, which are referred to as indels (Betton et al., 1993). Overall, these heterologous insertions introduced 4 to 19 aa residues, with an average net charge of −3 to +1, and several hydrophilic aa, with an average hydrophobicity of 0.25 to 3.55 (Table 1).

The indels ClpG 9/10.1 and ClpG 9/10.2, which contain a large disrupting aa sequence in the central hydrophobic core of SP, produced an anti-ClpG pAb-reacting 29-kDa protein comparable to the wt mature ClpG. In contrast, the hybrid ClpG monomers extracted from mutants ClpG 15/29.1 and ClpG 15/29.2, which are affected in the C-domain of the SP plus its 7 downstream aa, migrated slightly slower on SDS-PAGE than did the wt ClpG. In the indels ClpG 16/21.1 and ClpG 16/21.2 the four aa immediately preceding the SP-processing site, were deleted. These mutants revealed two protein bands of 29-kDa and 31-kDa that reacted with the specific anti-ClpG pAb on Western immunoblots. The amount of pilin in these mutants was low. The indels ClpG 21/22.1, ClpG 21/22.2 and ClpG 21/22.3C, containing a foreign peptide at the SP-mature ClpG junction, synthesized hybrid proteins whose sizes, as expected, were slightly greater than that of wt ClpG (Table 1).

Since in-frame substitutions in the wt cleavage site do not appear to affect the capacity of some indel pilins to reach the bacterial surface, processing must occur at some
Fig. 4. Analysis of the hybrid ClpG-TGEV-C epitope protein. (A) Synthetic oligo encoding the C epitope comprising aa residues 361-372 of the spike S protein of the porcine Purdue-115 strain of TGEV. The site C in a linear neutralizing epitope (Delmas et al., 1990). The mAb 3b.5 raised against TGEV and which recognized the C epitope on native virion (Laude et al., 1986) was used for the accurate screening of the hybrid ClpG-TGEV-C epitope construct. (B) Western blot analysis. Plasmids pGETA2, carrying the gene fusion encoding the hybrid ClpG::TGEV-C epitope protein, and pDEV41155, containing only the wt ClpG gene, were expressed in E. coli DHS[pDSPH524]. Cotransformants were grown overnight on LB agar medium with the appropriate antibiotics. Growing cells were scraped and resuspended in 1 ml of PBS (pH 7.2). The bacterial suspension was then vigorously agitated for 1 min with a top mix shaker, and placed at 60°C for 20 min. After centrifugation the supernatant containing the thermo-eluted limbrae was collected. Proteins present in the supernatant fraction were separated by 10% PAGE under denaturing conditions. Samples in Laemmli sample buffer were either boiled for 5 min in the presence of SDS and β-mercaptoethanol or incubated at room temperature for 15 min in the absence of SDS and β-mercaptoethanol to obtain the monomeric (a) and multimeric (b) forms of the proteins, respectively. Western blots were performed by the method of Towbin et al. (1979), with either the murine mAb 3b.5 or a rabbit anti-ClpG pAb, as primary Ab, and either a goat anti-mouse or a goat anti-rabbit IgG conjugated to peroxidase, as secondary Ab. Sheets were stained with 4-chloronaphtol. Lanes: 1, hybrid ClpG proteins; 2, wt ClpG proteins; (+) and (−) refer to the presence or the absence of TGEV C epitope, respectively. The 29-kDa and 31-kDa bands, corresponding to the wt ClpG and hybrid ClpG proteins, respectively, are indicated. (C) Immuno-EM of DHS[pGETA2, pDSPH524] producing CS31A::TGEV-C hybrid limbrae. Cells were cultured to the mid-exponential phase of growth, collected by brief centrifugation and resuspended in PBS (pH 7.2). Cells were then labeled on EM grids with mAb 3b.5 and 10-nm colloidal gold-labeled goat anti-mouse Ab. Gold labeling of intact cells was carried out essentially as described by Girardeau et al. (1988). The grids were EM examined with a Philips EM-400.
Characterization of ClpG indels

| Mutant ClpG | Indel length | ClpG size | Net charge | Average hydrophobicity |
|-------------|--------------|-----------|------------|-----------------------|
| ClpG 9/10.1 | 19           | 29        | -3         | -0.25                 |
| ClpG 9/10.2 | 19           | 70        | +1         | -2.00                 |
| ClpG 15/29.1| 5            | 30        | -3         | -0.75                 |
| ClpG 15/29.2| 5            | 30        | +1         | -2.71                 |
| ClpG 16/21.1| 14           | 29, 31    | 0          | -3.55                 |
| ClpG 16/21.2| 14           | 29, 31    | +1         | -2.88                 |
| ClpG 21/22.1| 18           | 32        | -3         | -0.93                 |
| ClpG 21/22.2| 18           | 32        | +1         | -2.76                 |
| ClpG 21/22.3C| 18         | 37        | -3         | -0.99                 |

* Mutants ClpG are designated by two slash-separated aa numbers corresponding to the N-terminal (first number) and C-terminal (second number) positions of the indel sites in the ClpG aa sequence relative to the N-terminal Met. The dot-following number indicates the identification order of mutants having a foreign sequence inserted at the same positions in ClpG (for example, ClpG 21/22.3 designates the third mutant selected for an insertion between the aa 21 and the aa 22 of ClpG). The presence of TGEV-C epitope is indicated by the letter C after the dot-following number (ClpG 21/22.3(3).

** Table I

Indel mutation represents insertion with or without deletion, and indel length, i.e., the net length resulting from these insertions. The deletion (−) and insertion (+) lengths, in parentheses, are expressed in the number of aa with respect to wt ClpG.

* The size of the mature ClpG protein expressed in kDa, as determined by SDS-PAGE and Western immunoblotting with specific ClpG pAb, as described in the legend to Fig. 4.

* Net charge exhibited by the heterologous aa sequence is given by counting +1 for Arg (R) and Lys (K), and −1 for Asp (D) and Glu (E). The numbers of acidic (−) and basic (+) aa residues distributed in the inserted peptide are indicated in parentheses on the left and on the right, respectively.

* Average hydrophobic index of inserted peptides in the ClpG sequence environment. Hydrophobic indices were calculated from the hydrophobicity scale of Eisenberg et al. (1984), with an 11-aa window and vectors projecting radially every 100°. The average hydrophobic index was calculated as the arithmetic mean of the hydrophobic index of the 11-aa-long peptides.

Fig. 6. SP aa sequences of fimbrial components members of the CS31A-related family. (A) Comparison and alignment of the SP aa sequences of the major ClpG, FaeG and F41 prepilins, and of the OmpA outer membrane protein. Gaps were introduced to obtain maximal fitting. Identical aa and functionally similar aa from consensus sequence are in bold and normal characters, respectively. The meaning of the capitalized letters N, H and C below the consensus sequence is indicated in the Fig. 3 legend. The cryptic consensus Ser-flanked sequences are underlined. (B) Analysis of the C-terminal part of the SP of nine K88 and CS31A accessory proteins. FaeD, FaeE, FaeF, FaeH and Fael are involved in the K88 fimbria biosynthesis (Bakker, 1991), whereas ClpE, ClpF, ClpH and ClpI refer to the CS31A system (Martin et al., 1991). FaeD functions in the translocation of the fimbrial subunits across the outer membrane (OM) and it may serve as an anchor protein. FaeE and ClpE are periplasmic (P) chaperones. FaeF, ClpF, FaeH, ClpH, FaeI and ClpI are minor fimbrial subunits (nPi) which function in initiation, elongation and/or termination of fimbrial formation. The serine-flanked sequences in large bold characters are underlined. The small bold numbers indicate the aa positions with respect to the full signal aa sequences.

OmpA SP did not mediate its proper translocation across the cytoplasmic membrane was strengthened by Western blot analysis with the anti-FLAG peptide M1mAb. This mAb specifically binds to the eight aa FLAG peptide (DYKDDDDK; Fig. 7A) only when it is located at the free N terminus of a FLAG fusion protein, but does not bind to an unprocessed N-terminal FLAG fusion protein (Prickett et al., 1989). Thus, given that the fusion OmpA–ClpG protein did not react against M1mAb (Fig. 7B), and from the data reported above, we conclude that the OmpA SP was unprocessed.

Alignment and comparison of the SP aa sequences of ClpG, FaeG and F41 prepilins with that of OmpA (Fig. 6A) showed a cryptic consensus sequence, namely S-A-(A)-V/A-S, only at the C-terminal end of the ClpG, FaeG and F41 hydrophobic cores. It contains polar Ser, hydrophobic Ala and Val residues, which form an α-helix. Previous studies have pointed out the importance of an α-helical conformation in the hydrophobic region for SP function (Pugsley, 1993). In particular, the controlling factor seems to be the overall propensity for helix forma-
That this phenomena is not applicable to OmpA::ClpG fusion, since it was unprocessed in spite of a normal α-helix structured OmpA SP, implies that conformation of the SP may be influenced by the mature region. An earlier study (Girardeau et al., 1991) showed a substantial sequence similarity both between the mature regions of ClpG, FaeG and F41 fimbrial subunits and between their corresponding SP. These findings suggest that the importance of a given aspect of SP structure depends on the associated mature region. Thus, in ClpG-related pilins, the conformational properties of aa residues composing the S-A-(A)-V-A-S sequence might either (i), play a role in the above association by making the normal cleavage site more accessible to the peptidase, depending on the aa sequences in the vicinity of the site, or (ii), act as a topologic signal by which nascent subunit molecules are recognized for exportation across the cytoplasmic membrane by a specialized export machinery homologous to CS31A. K88 and F41 systems and capable of positioning the prepilin for the cleavage event within the membrane in association with one of CS31A accessory proteins. The CS31A fimbrial ClpE chaperone, required for the transport of ClpG across the periplasm (Bertin et al., 1993), appears as a good candidate capable of interacting with both ClpG SP and mature ClpG. Indeed, Hultgren et al. (1989) reported that processing of the PapG adhesin by signal peptidase I is enhanced by its interaction with the PapD pilin chaperone, and that the last 13 aa at the C-terminal part of the mature PapG were directly involved in the PapG-PapD complex formation. Fimbriae are usually composed of several different subunits, all of which are recognized by the same molecular chaperone. If our second hypothesis is true, the specific mechanism by which the membrane translocation of the major fimbrial subunits occurs through the Ser-flanked sequence recognition by ClpE, must be also applicable to other exported proteins members of the fimbrial assembly machinery. In agreement with this reasoning, most SP aa sequences of nine CS31A and K88 accessory proteins revealed a Ser-flanked segment of 4-7 aa residues near the peptidase cleavage site (Fig. 6B). This segment was missing only for FaeE and ClpE chaperones, strengthening the view that ClpE could be involved in the Ser-flanked sequence recognition. Since the Ser-flanked part of SP is always very closed to the normal peptidase recognition sequence. ClpE binding to this part of SP would authorize the accessibility of cleavage site to signal peptidase I.

Conclusions

(1) Integrity of the CS31A fimbrial ClpG subunit-related SP is not strictly required for pilin assembly. Indeed, despite the extensive modifications in the signal sequence region, the mutant ClpG pilin was processed in every case. In particular, the normal peptidase recognition sequence is dispensable since major substitutions of residues across this processing junction were permissive for CS31A fimbria production. Although this is the first

Fig. 7. Construction and expression of the hybrid ompA::clpG gene. (A) In-frame fusion between ompA signal sequence and clpG gene is shown. The wt mature ClpG-encoding sequence was removed by EcoRI-XbaI double digestion of pGETA2 from ClpG 21/22::C mutant (section c., legend to Fig. 4 legend and Fig. S), and inserted into EcoRI +XbaI-linearized pFLAG-1™ (International Biotechnologies) to give the plasmid pOPA31. The nt and aa sequences from the ompA and ClpG sequences are in small characters. The boxed designations, SPOmpA and ClpG, symbolize the nt and aa sequences corresponding to OmpA SP and ClpG mature protein, respectively. (B) E. coli DH5α cells harboring either, pDEV41120 (Lane 1), or complemented pDEV41125 (Lane 2), or complemented pOFA31 (Lane 3), or pDPSH524 (Lane 4), were grown at 37°C in LB medium (5 ml) supplemented with appropriate drugs and 1 mM IPTG if necessary (only for the fusion construct). When they reached an A600 of 2.0 (for cells containing fusion, IPTG was added at an A600 of 0.7), the cells were collected by centrifugation, resuspended in water (20 μl), and diluted twofold in loading buffer (60 mM Tris pH 6.8, 10% glycerol, 2% SDS, 100% β-mercaptoethanol). The suspension was then boiled for 5 min and run on a 10% SDS-10% PA gel. Western immunoblot analysis, with either a rabbit anti-ClpG pAb (a) or the murine anti-Flag peptide M1 mAb (b) was performed as described in the legend to Fig. 4B. m and p indicate wt mature protein and hybrid precursor protein, respectively.
time these findings have been reported for an E. coli pilin, similar observations were made with Pseudomonas aeruginosa type-IV pilin leader-peptides mutants (Strom and Lory, 1991).

(2) In agreement with the conclusions of Hemilä et al. (1992), fusions containing intact SP, which are common when producing heterologous proteins, are not necessarily the most suitable.

(3) The work reported here suggests a specific functional interrelationship of the ClpG SP with its associated mature region. A possibility is that the mature region and the SP interact either directly through definite aa zones of recognition, or indirectly in association with other components of the CS31A fimbria assembly machinery.

(4) It is possible to insert a viral antigenic determinant into CS31A fimbriae in such a way that it is cell-surface exposed and that intact recombinant cells are recognized by a mAb directed against the viral epitope. This opens up the possibility of creating diagnostic reagents based on whole cells, of developing new live vaccines and of producing foreign polypeptides.

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