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Protein secretion in *Escherichia coli* K-12: dead or alive?

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Abstract. *Escherichia coli* K-12 possesses a large number of chromosomal genes that, in other Gram-negative bacteria, are involved either in exoprotein secretion or in the formation of type IV pili. Some of these *E. coli* genes have been shown to encode proteins when expressed from heterologous promoters. Furthermore, at least two of these proteins are functional in heterologous complementation tests, but none of the genes examined so far is expressed when *E. coli* is grown under standard laboratory conditions. We propose that transcription of these genes is turned off during growth in laboratory medium, that their expression is controlled by environmental sensor proteins and that they could play an important role in pathogenicity or in the utilization of large polymers.

Key words. Protein secretion; pili; outer membrane; general secretory pathway; secreton.

Secretion and piliation in Gram-negative bacteria

Gram-negative bacteria possess several pathways for the localization of proteins to extracytoplasmic compartments or the growth medium [1]. One of these, the general secretory pathway (GSP), involves a common step for the translocation of periplasmic and outer membrane proteins across the cytoplasmic or inner membrane (the signal peptide-dependent Sec pathway [2]), followed by a large number of alternative terminal branches that direct exoproteins across the outer membrane [2]. These exoproteins may be released into the growth medium, remain anchored to the cell surface or form filamentous cell surface appendages called pili.

Our studies are aimed at elucidating two of these terminal pathways. One of them is the main terminal branch for exoprotein secretion in Gram-negative bacteria. In the specific example of this pathway that we have been studying, 14 gene products are required for the secretion of a single enzyme, pullulanase, by *Klebsiella oxytoca*. These 14 proteins are proposed to be involved in the assembly of a still hypothetical membrane structure called the secreton that directs the selective, energy-dependent translocation of folded polypeptides across the outer membrane. Putative secretion signals in the pullulanase polypeptide have been identified [3], functions have been assigned to or proposed for some of the 14 secretion gene products [4–8] and the energy source needed for translocation has been determined [9]. Similar systems involving closely related gene products have been identified in many other Gram-negative bacteria; indeed, some of the secreton components are interchangeable, at least between closely related species, even though the proteins secreted by one bacterium are often not secreted by its close relatives [10–12].

Our approach to the study of pullulanase secretion capitalized on the fact that it could be fully reconstituted in *Escherichia coli* K-12. Knock-out mutations in each of the 14 pullulanase secreton genes prevented secretion of the enzyme in *E. coli* [13–15], implying that this bacterium is a neutral host that either does not contain homologues of the secreton genes or, alternatively, does not express them. In fact, *E. coli* K-12 possesses homologues of 13 of the 14 pullulanase secreton genes [16, 17], and two of them at least can replace the corresponding genes of the pullulanase secreton

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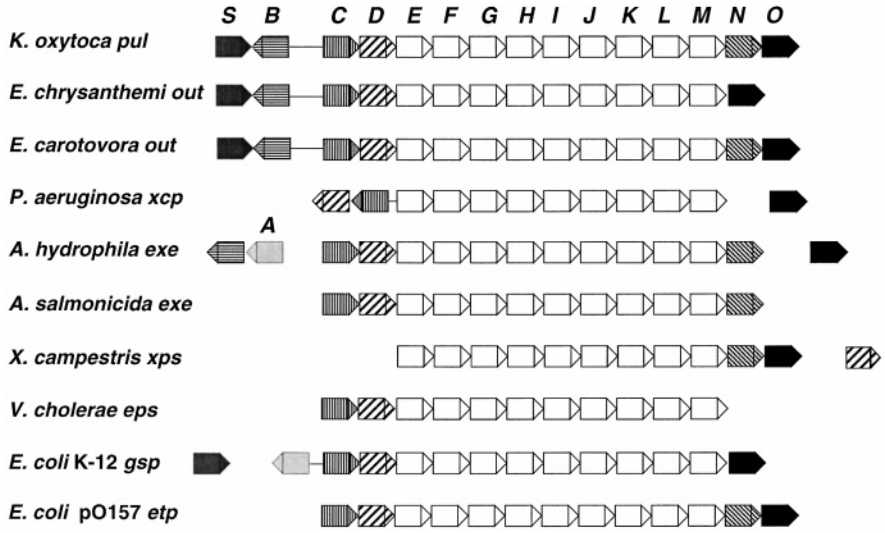

Figure 1. Schematic representation of chromosomal genes coding for components of the secreton of the GSP in the Gram-negative bacteria *Klebsiella oxytoca*, *Erwinia chrysanthemi*, *E. carotovora*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *A. salmonicida*, *Xanthomonas campestris*, *Vibrio cholerae*, *Escherichia coli* K-12 and the plasmid-borne genes in *E. coli* O157. Genes are represented as boxes with arrowheads indicating their orientation, and are identified by the fourth letter of their standard designation (the first three letters are indicated at the left). The nomenclature of the *xcp* genes is different; the *O* gene is called *xcpA* or *pilD* and the other genes are called *xcpP* through *xcpZ*. A new, standard nomenclature is being progressively introduced for the *xcp* genes. Boxes that are not shaded indicate homologous genes in the same position in different species. Note, however, that some strains apparently lack the *N* gene and that the *A* gene has so far been found in only two bacteria (*E. coli* K-12 and *A. hydrophila*). These and other genes whose orientation and/or position differs in different bacteria are shaded. The *pulB* gene is not required for pullulanase secretion in *E. coli* K-12 carrying the entire gene cluster [37; 38], but its homologues *outB* and *exeB* are required for protein secretion in *E. chrysanthemi* [39] and *A. hydropila* [40], respectively. Continuous thin lines between genes indicate relatively short intervening stretches (containing genes for exoproteins and/or divergent promoters), and spaces indicate that the genes are not adjacent. The figure, which is not to scale, was compiled from unpublished information from G. Salmond and from published data and sequences in Genbank [13–15, 22, 37–49].

when they are cloned and expressed from a heterologous promoter [17].

As in most other, related secreton systems, 13 of the 14 pullulanase secreton genes are located in a single operon (fig. 1). This is also the case for the corresponding *gsp* genes in *E. coli* K-12, except that the *N* gene, present in *K. oxytoca*, is missing from *E. coli* K-12 (Fig. 1). The fourteenth gene, *S*, is located close to the secreton operon in *K. oxytoca* but is located at a different map location (2.95 min on the circular chromosome) from the secreton operon (74.5 min) in *E. coli* K-12 [16, 18]. The *S* gene has so far not been found in other bacteria except *Erwinia* (fig. 1). Another important difference between the *E. coli* secreton genes and those in some other bacteria is the presence of a gene, *gspA*, that has hitherto only been found in *Aeromonas hydrophila*, where it is involved in, but is not absolutely required for, secreton function [19].

In *E. coli* K-12, the *gspA* gene and the *gspC* gene, the first gene of the secreton operon (fig. 1), face in opposite directions and are separated by 180 bp that are presumed to contain regulatory elements common to both genes. Operon fusion studies demonstrated that neither promoter is active under standard laboratory conditions [17], in line with the fact that *E. coli* K-12 does not secrete exoproteins. However, sequence analysis of the *E. coli* K-12 secreton genes did not reveal any obvious deletions or insertions (except for the absence of an *N* gene) or stop codons in the predicted reading frames [16]. This is not what one might expect for genes that have been dormant for a long period of time. Indeed, as already mentioned, two of the *E. coli* secreton genes, *G* and *O*, encode functional proteins [17]. These observations suggest that the promoter of this secreton operon, while inactive in laboratory media, might be activated under specific environmental conditions. If this is the case, the operon might be regulated by an environmentally sensitive regulator, which could either be a transcription activator or a repressor that is specific for this promoter, or a global regulator affecting several different promoters.

Several experimental approaches have been used to detect such regulators. First, the promoter region was cloned on a high-copy-number plasmid that would be expected to titrate any specific repressor protein that controlled its expression. Introduction of this plasmid into a strain carrying a *gspC-lacZ* operon fusion did not increase β-galactosidase levels, suggesting that the putative regulator is not a repressor [17]. Next, we examined the ability of H-NS, a global regulator with histonelike properties, to bind to the promoter. H-NS bound very strongly, as revealed by band-shift experiments [19a]. A mutant lacking H-NS protein was therefore examined for its level of *gspC-lacZ* expression. β-galactosidase levels increased to two to three times their normal level in these strains. However, examination of culture supernatants failed to reveal any evidence of specific protein secretion, because the cells leaked periplasmic proteins nonspecifically into the growth medium. Furthermore, although *gspC* promoter expression was increased by the *hns* mutation tested, the levels reached were still very low.

Thus, we have so far failed to find a gene that could exert a dramatic effect on the expression of the *E. coli* K-12 secreton genes. Furthermore, none of a wide variety of growth conditions (variations in temperature, pH, osmolarity, ion content, serum levels) was found to affect *gspC-lacZ* expression. Finally, an antiserum raised against *E. coli* K-12 GspG protein failed to detect this protein in several non-K-12 *E. coli* isolates grown in rich medium at 37 °C. However, many of them were found to possess a copy of the *gspO* gene, indicating that the entire *gsp* secreton operon probably exists in these strains [19a]. Thus, the role of the *E. coli gsp* genes remains enigmatic.

Wider still and wider

The last gene of the *E. coli* K-12 *gsp* operon, *gspO*, codes for a prepilin peptidase that, in other secreton systems, cleaves and *N*-methylates four other secreton components, G, H, I and J (the pseudopilins [6, 20–22]). The product of the *O* gene is called prepilin peptidase because it is homologous to the enzyme that processes the precursors of type IV pilins in exactly the same way [23], allowing them to assemble into pili. The sequences around the cleavage sites in the pilin and pseudopilin precursors are almost identical [24]. In *Pseudomonas aeruginosa*, the same gene product performs both functions [22, 23]. We had previously observed partial processing of one particular prepilin peptidase substrate, the type IV major pilin subunit (PilE) of *Neisseria gonorrhoeae*, when its structural gene was expressed in *E. coli* K-12, and we hypothesized that this processing might be carried out by the product of a prepilin peptidase gene. Subsequent studies demonstrated that prePilE was not the only potential prepilin peptidase substrate that could be processed, that processing only occurred in cells grown at 37 °C or higher and that it required the presence of a consensus prepilin peptidase cleavage site and was unaffected by mutations affecting several well-characterized, nonspecific proteases [19a]. The most obvious candidate prepilin peptidase gene, *gspO*, was ruled out both by the fact that the gene is not expressed and by the fact that a knock-out mutation in *gspO* did not prevent prePilE processing [17]. This led us to search for a second prepilin peptidase gene in the *E. coli* K-12 genome data bank. Such a gene was found as part of a cluster of genes at 67 min on the *E. coli* K-12 chromosome that appears to be a disorganized secreton operon or a set of genes involved in type IV piliation [19a]. The gene, named *pppA*, was found to encode a fully functional prepilin peptidase and, furthermore, was found to be responsible for the processing of prepilin peptidase substrates in *E. coli* K-12 [19a]. Nevertheless, the gene is poorly expressed, and, surprisingly, its transcription is not higher at 37 °C than at 30 °C, a temperature at which prepilin peptidase activity cannot be detected in *E. coli* K-12.

This is the first time that a bacterium has been shown to possess two chromosomal prepilin peptidase genes, which is quite remarkable considering that *E. coli* K-12 is not known to secrete proteins or to produce type IV pili. Nevertheless, the *E. coli* K-12 chromosome does have homologues of genes that, in other bacteria, are involved in the assembly of type IV pili [25]. Many of these genes are homologous to secreton genes, reflecting the close evolutionary relationship between piliation and secretion and the fact that the secreton might be some form of rudimentary pilus whose assembly therefore involves components similar to those involved in pilus formation. This makes it difficult to sort out the exact function of the *pppA*-encoded prepilin peptidase. Our current working hypothesis is that its main role is to process the precursors of the type IV pilins. We therefore examined the expression of other genes that might be involved in type IV piliation, most notably three clustered genes [25], one of which, *ppdD*, appears to code for a prepilin, while the other two, *hopB* and *hopC*, appear to code for proteins closely related to factors needed for type IV piliation in, for example, *P. aeruginosa* [26] and *N. gonorrhoeae* [27–29]. Expression of all three *E. coli* genes, which appear to form an operon [25], under the control of the T7 bacteriophage gene *10* promoter resulted in the production of three proteins whose sizes were close to those predicted for

the *ppdD*, *hopB* and *hopC* gene products (fig. 2). Furthermore, the predicted *hopB* gene product formed intramolecular disulphide bonds, as observed previously for its homologue PulE [10]. Finally, the *ppdD* gene product was processed in cells expressing *pppA* [19a].

Thus, *E. coli* K-12 has a set of type IV piliation genes that are not interrupted by stop codons and so, like the secreton genes, are likely to be dormant but not dead. *E. coli* K-12 has never been reported to produce type IV pili, although some plasmid-bearing *E. coli* strains do possess type IV pili [30–33]. Are the piliation genes expressed in E. coli K-12 or can their expression be induced in any way? We have only just started to try to answer these important questions. First, we screened a number of wild *E. coli* isolates by Southern hybridization and polymerase chain reaction (PCR) for genes homologous to *pppA* and *ppdD*. Both genes were found to exist in the majority of the strains tested; in fact, *ppdD* was found in all 16 strains tested, some of which did not have *pppA* [19a]. Next, we raised antibodies against the *ppdD* gene product and used them to screen all of these strains, including *E. coli* K-12, for the presence of the protein under routine laboratory conditions [19a]. None of the strains produced a protein that reacted with the antibodies. Clearly, more work has to be done to unravel the interrelationships between the secreton and piliation genes and to study their individual and possibly coordinate regulation.

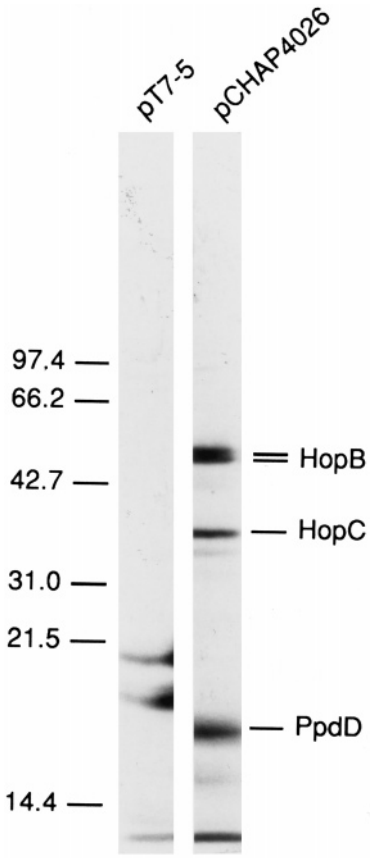

Figure 2. Expression of the *ppdD*, *hopB* and *hopC* and genes from the bacteriophage T7 gene *10* promoter. The genes were present on a PCR-amplified DNA fragment obtained using the *E. coli* K-12 chromosome as a template. The autoradiograph shows ^{35}[S]-methionine-labelled proteins produced by cells carrying the vector, pT7-5 [50], or the vector carrying the cloned genes (pCHAP4026). Production of the bacteriophage T7 RNA polymerase was first induced by adding isopropyl β-D-thiogalactoside (IPTG) to the culture (the polymerase gene in the strain used is under the control of the IPTG-inducible *lacZ* promoter), and then rifampicin was added to prevent transcription by *E. coli* RNA polymerase before the radioactive methionine was added to label the proteins synthesized. Positions of the molecular size markers (kD) are indicated.

Dead or alive?

The success of our attempts to determine the function of the *E. coli* K-12 secreton or type IV piliation genes hinges on our ability to find conditions under which they are expressed. This strategy is not without risk; for example, it may be that the genes in strain K-12 have lost their ability to be expressed as a result of its prolonged storage and passage in the laboratory. The putative regulator of the secreton operon might no longer exist in this bacterium, for instance, whereas it might still be present and functional in more recently isolated strains. Unlike the secreton genes, the piliation genes are scattered in small clusters throughout the chromosome. Therefore, we need to examine every cluster separately to determine whether any of them contain genes that are expressed. If some gene clusters are expressed while others are not, then the nonfunctioning promoters will be replaced by heterologous promoters, such as *lacZp*. A similar strategy is currently being employed in an attempt to express *gspA* and the *gspC-O* operon.

What will happen when the secreton and piliation genes are expressed? Obviously we would hope that the cells would secrete something or would assemble type IV pili. What type of protein might be secreted? One possibility is that it would be a protein that resides in the periplasm in laboratory-grown *E. coli* K-12. There is, in fact, a precedent for an exoprotein that is not secreted by the natural *E. coli*-producing strains: some clinical isolates produce a periplasmic heat-labile enterotoxin (HLET) that is closely related to cholera toxin (CT). *Vibrio cholerae* secretes CT by the secreton pathway, and it can also secrete HLET. However, we did not find any homologues of the genes that code for HLET/CT

subunits in the *E. coli* K-12 genome. Nevertheless, it remains possible that *E. coli* K-12 has an uncharacterized periplasmic toxin or an enzyme that, under some circumstances, should (and can?) be secreted extracellularly.

Alternatively, expression of the gene(s) coding for the secreted protein(s) might be coordinated with that of the secreton genes. In this case, replacing the intragenic *gspA-gspC* region with a heterologous promoter capable of being expressed would not lead to protein secretion. It is intriguing that the recently discovered gene for the *E. coli* K-12 hemolysin is repressed by HNS [34], as is the *gspC* promoter [19a]. However, the hemolysin does not have a signal peptide [34] and is therefore unlikely to be secreted by the secreton.

The situation with regards to the type IV pili is equally interesting. Type IV pili are usually associated with pathogens and, in some cases, have been shown to help the bacteria to adhere to mammalian cells [35, 36]. If this is the case with the *E. coli* type IV pili, it is surprising that the gene coding for the putative major pilin (*ppdD*) is present in all *E. coli* strains tested, since not all *E. coli* strains are pathogens. An alternative role for these pili could be in the interaction of the bacterium with inert surfaces outside the intestinal tract. It is quite conceivable that the secreton and piliation genes are coordinately expressed, because they are all involved in a similar process, whether it be pathogenicity or polymer degradation. Whatever the results of our investigations, it is clear that *E. coli* possesses hitherto unidentified properties that are likely to be important to it under certain environmental conditions and that deserve more thorough investigation.

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