The Cryptic General Secretory Pathway (gsp) Operon of Escherichia coli K-12 Encodes Functional Proteins

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Systematic sequencing of the Escherichia coli K-12 chromosome (GenBank entry U18997) has revealed the presence of an apparently complete operon of genes (the gsp-C-O operon) similar to genes coding for components of the main terminal branch of the general secretory pathway (e.g., the Klebsiella oxytoca pulC-O pullulanase secretion operon) and to related genes required for type IV pilus biogenesis. For example, the last gene in the gsp operon, gspO (formerly hopD), encodes a protein which is similar to several type IV prepilin peptidases. Expression of gspO from lacZp promotes cleavage of two known prepilin peptidase substrates in E. coli K-12: Neisseria gonorrhoeae type IV prepilin and K. oxytoca prePulG protein. gspO also complements a mutation in the corresponding gene (pulO) of the pululanase secretion operon when it is expressed from lacZp. Another gene in the gsp operon, gspG (formerly hopG), encodes a protein similar to prePulG, a component of the pullulanase secretion pathway. Expression of gspG from lacZp leads to production of a protein which (i) is recognized by PulG-specific antiserum (and by antiserum against the Pseudomonas aeruginosa PulG homolog XcpG [formerly XcpT]), (ii) is processed in cells expressing gspO, and (iii) restores secretion in cells carrying a pulG mutation. The chromosomal copies of gspG and gspO are apparently not expressed, probably because of very weak transcription from the upstream region, as measured by using a chromosomal gspC-lacZ operon fusion. Thus, the gsp operon of E. coli K-12 includes at least two functional genes which, together with the rest of the operon, are probably not expressed under laboratory conditions.

Several species of gram-negative bacteria secrete extracellular proteins via a two-step general secretory pathway (GSP) which begins with Sec-dependent, signal peptide-mediated translocation across the cytoplasmic membrane. Subsequent steps of specific recognition and transport across the outer membrane involve components of the main terminal branch (MTB) of the GSP (20). In one of the most intensively studied of these systems, the pullulanase secretion pathway of Klebsiella oxytoca, the roles of 2 of the 14 proteins (PulS and PulO) which make up the MTB have been determined. PulS is a chaperone-like protein which facilitates the insertion of another MTB component, PulD, into the outer membrane (8). PulS is encoded by a constitutively expressed gene (3), whereas PulD is encoded by the second gene in the 13-gene, maltose-inducible, MalT-regulated pulC-pulO operon (4). The last gene in this operon, pulO, codes for an enzyme required for the processing of another protein encoded by the pulC-pulO operon, PulG (21, 23), and, by analogy with the related secretion system in Pseudomonas aeruginosa (16), of three other proteins, PulH, PulI, and PulJ. The processing site in these proteins is similar to that in precursors of type IV pilins (24). The latter are cleaved and methylated by enzymes called prepilin peptidases (7, 12, 13) whose amino acid sequences are similar to that of PulO (20). Genes coding for the prepilin peptidases of P. aeruginosa and Neisseria gonorrhoeae can complement mutations in the pulO gene (7).

Most studies of pullulanase secretion have been performed with Escherichia coli K-12 carrying the cloned pul genes, including the pullulanase structural gene (pulA), integrated into the chromosome or on plasmids. It was assumed that E. coli K-12 did not possess pul gene homologs since this bacterium is not known to secrete extracellular proteins and since pulE- and pulO-specific probes failed to hybridize with restricted E. coli K-12 chromosomal DNA under high-stringency conditions (6, 19). However, Andrews et al. (1) identified and sequenced part of a gene which could code for a protein with sequence similarity to PulO during their studies of the adjacent bacterioferritin structural gene in the E. coli chromosome. Whitchurch and Mattick (30) subsequently showed that this gene (which they called hopD for homolog of pilin gene D because of its similarity to the P. aeruginosa prepilin peptidase gene pilD) was complete and was nonessential. Shortly thereafter, Stojiljkovic et al. (28) sequenced a gene, which they called hopG, which is very similar to pullG and which is flanked by homologs of the pulF and pulH genes. When expressed under the control of a bacteriophage T7 promoter, hopG was shown to encode a ca. 17-kDa protein (28). These genes were shown to be located just upstream from and in the same orientation as hopD. Finally, the complete sequence of this region of the E. coli K-12 chromosome (min 73) revealed the presence of homologs of all of the genes in the pulC-O operon except pulN in what appears to be an uninterrupted operon (17). In view of the closer similarity of these E. coli genes to the genes coding for GSP-MTB components than to genes required for type IV piliation, we propose that they be renamed gsp (for general secretory pathway). Thus, hopF, hopG, hopH, and hopD become gspF, gspG, gspH, and gspO, respectively. The organization of the gsp genes in the E. coli K-12 chromosome is represented schematically in Fig. 1. The first gene in the operon, gspC, is preceded by the divergently transcribed gene gspA. This gene is homologous to the Aeromonas hydrophila exeA gene, which also codes for a component of the GSP-MTB (10).

To date, the only documented evidence that any of the E. coli gsp operon genes had been expressed in E. coli K-12 is the demonstration that hopD and hopG complement pulO and pulG mutations, respectively. Therefore, we propose that the gsp operon be renamed hop (for homologous to E. coli pil operon). Finally, it is of interest that the products of gspF, gspG, gspH, and gspO are similar to the products of the prepilin peptidase genes of P. aeruginosa, which are involved in pilus biogenesis (27). Thus, it can be postulated that E. coli has a cryptic general secretory pathway (GSP-MTB) similar to that of MTB, which is expressed under extreme conditions. If this is true, then the E. coli chromosome could be considered to be nearly complete [as in the case of the chromosome of Klebsiella oxytoca (9)]

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coli K-12 gsp genes could be functional is that expression of the N. gonorrhoeae gene for the precursor of type IV pilin in E. coli K-12 results in the production of pilin which comigrates with mature pilin, possibly because of processing by gspO (hopD)-encoded preplin peptidase (7, 12). However, another potential substrate for the GspO preplin peptidase, PulG, is not cleaved when produced in E. coli K-12 (21, 23). To evaluate the expression and functionality of gspO and of the pulG homolog gspG, we expressed these genes under the control of a lacZ promoter on plasmids and tested their ability to complement pulO and pulG mutations, which prevent pullulanase secretion. We also tested the effects of a mutation in the chromosomal gspO gene (30) on prepil processing. Finally, we inserted the region encompassing the gspA-gspC intergenic region into a promoter probe vector to test for the presence of potential promoters.

**MATERIALS AND METHODS**

**Plasmids, bacterial strains, and growth conditions.** The main characteristics of plasmids used in this study are given in Table 1. The gspG gene was amplified from E. coli K-12 chromosomal DNA by using a pair of primers that hybridize with 17 bp within the 3' end of the preceding gene (gpf) or within the 5' end of the following gene (gph). The primers introduced a unique EcoRI site at the 5' end of the amplified fragment and a unique HindIII site at its 3' end. The amplified DNA was cleaved with EcoRI and HindIII and cloned into the corresponding sites of pUC18 to give pCHAP4010 (Table 1). The insert in pCHAP4043 (Table 1) was derived from pCHAP4010. The gspA-gspC promoter region was amplified in a similar way by using primers that hybridize with sequences within the 5' coding sequences of the gspA and gspC genes. The primers introduced unique BamHI sites at both ends of the amplified fragment, which was cloned into the BamHI site of pUC18 (to give pCHAP4051) and thence into pRS551 to give pCHAP4048 and pCHAP4053 (Table 1).

The E. coli strains used as hosts for most of the experiments involving plasmids were MC4100 and its derivatives or PAP105 (Table 2). Strains with single-copy insertions of the gspA-lacZ or gspC-lacZ operon fusions were constructed by lysogenizing with bacteriophage λ recombinants as described by Simons et al. (27).

Bacteria were generally grown in Luria-Bertani (LB) broth (14) with vigorous shaking at 30°C (or at 37°C when processing of prepilE in the absence of exogenous prepilin peptidase was to be studied). When required, antibiotics were added at the following concentrations: ampicillin, 100 µg/ml; chloramphenicol, 25 µg/ml; tetracycline, 16 µg/ml; and kanamycin, 50 µg/ml. For cells in which expression of the pulG gene was induced by maltose (0.4%), the medium was buffered by the addition of 10 mM phosphate buffer (pH 7.1). Where indicated in the figures, isopropyl-1-thiogalactopyranoside (IPTG) was used at 1 mM to induce expression of genes under lacZ promoter control.

**Protein analysis and enzyme assays.** Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on Tris-glycine-buffered gels containing 8 M urea as described previously (21, 23). After electrophoresis, proteins were transferred onto sheets of Schleicher and Schuell BA83 nitrocellulose in a semidry blot apparatus. The primary antibodies used were a PulG-specific antiserum (23) diluted 1:2,000, an antiserum raised against XcpG (for-

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**TABLE 1. Plasmids used in this study**

| Plasmid   | Characteristics                                                                 | Source or reference |
|-----------|---------------------------------------------------------------------------------|---------------------|
| pBShopD   | pBluescript carrying gspO (hopD) from pGS280 (1) under lacZp control (Apr')     | 30                  |
| pCHAP4015 | pHSGB756 (28) carrying gpf cloned from pBShopD as an XhoI-BamHI fragment under lacZp control (Cm') | This study          |
| pCHAP155  | pHSGB75 carrying pulO under lacZp control (Cm')                                   | 23                  |
| pCHAP158  | pEMBL85 carrying pulO under lacZp control (Apr')                                 | 23                  |
| pCHAP4010 | pUC18 carrying PCR-amplified gpf gene under lacZp control (Apr')                 | This study          |
| pCHAP4043 | pACYC184 carrying PCR-amplified gpf gene cloned under control of the constitutive cat promoter (Tc') | This study          |
| pCHAP162  | pBGS197 carrying pulG under lacZp control                                        | 23                  |
| pNG1100   | pBR322 carrying pilE cloned from N. gonorrhoeae MS11 (Apr')                      | M.-K. Taha         |
| pNG300    | pGZ carrying pilE cloned from N. gonorrhoeae MS11 (Cm')                          | 7                   |
| pRS551    | lacZ operon fusion promoter probe vector (Km')                                   | 27                  |
| pCHAP4053 | pRS551 carrying putative gspA-gspC intergenic region cloned as a BamHI fragment to create a gspC-lacZ operon fusion (Apr') | This study          |
| pCHAP4048 | Same as pCHAP4053 but with fragment in reverse orientation to create a gspA-lacZ operon fusion (Apr') | This study          |
| pCHAP4051 | pUC18 carrying same BamHI fragment as pCHAP4053 (Apr')                           | This study          |

*Further details of plasmids constructed as part of the present study are given in Materials and Methods.*
merely XcpT (a gift from S. Lory) diluted 1:3,000, and a gonococcal pilin-specific antiserum (a gift from B. Dupuy) diluted 1:1,000. The secondary antibody was horseradish peroxidase-coupled anti-rabbit immunoglobulin G (Amersham) or alkaline phosphatase-coupled anti-rabbit immunoglobulin G (Promega) used as directed by the manufacturer, and the nitrocellulose membranes were developed by enhanced chemiluminescence with ECL reagents (Amersham) or directly by the manufacturer, and then nitrocellulose membranes were developed with horseradish peroxidase-coupled anti-rabbit immunoglobulin G (Amersham) or merely XcpT (a gift from S. Lory) diluted 1:3,000, and a gonococcal pilin-specific antiserum (a gift from B. Dupuy) diluted 1:1,000. The secondary antibody was horseradish peroxidase-coupled anti-rabbit immunoglobulin G (Amersham) or alkaline phosphatase-coupled anti-rabbit immunoglobulin G (Promega) used as directed by the manufacturer, and the nitrocellulose membranes were developed by enhanced chemiluminescence with ECL reagents (Amersham) or with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate, respectively. Cell surface presentation of pullulanase was determined by assaying the proportion of the enzyme which could be detected in whole cells compared with that in detergent-lysed cells as previously described (13, 21).

RESULTS AND DISCUSSION

GspO can process prePulG and prePilE. A plasmid (pBSHopD) carrying the gspO gene under lac promoter control in pBluescript SK+ was kindly provided by Whitchurch and Mattick (30) (Fig. 1). Introduction of pBSHopD into E. coli K-12 strain PAP7245, in which the entire pul gene region is inserted into the chromosome and in which pulO has been inactivated by a short, uncharacterized deletion (23), restored pullulanase secretion. Full restoration of pullulanase secretion was achieved only when gspO expression was increased by IPTG induction (Table 3). Similar results were obtained with pCHAP4015, a low-copy-number pHSG576 derivative (29) carrying gspO cloned from pBSHopD (data not shown). Furthermore, PulG produced by cells expressing gspO was found in an apparently processed form whose size was indistinguishable from that of PulG produced by cells expressing pulO (Fig. 2). Thus, like the N. gonorrhoeae pilD gene and the P. aeruginosa xcpA (pilD) gene (7), the E. coli K-12 gspO gene encodes a functional prepilin peptidase which can substitute for PulO in the processing of prePulG protein. It is important to note, however, that prePreUlG was not processed unless either pulO or gspO was present on a plasmid and that pulO mutations abolish pullulanase secretion completely, indicating that the chromosomal copy of gspO cannot substitute for pulO.

Similar tests were performed to determine whether the gspO gene product could cleave N. gonorrhoeae prepilin. In this case, the cells carried pNG300 (pilE) and either pCHAP158 (pulO) or pBSHopD (gspO). Immunoblot analysis with antiserum raised against gonococcal pilin revealed IPTG-dependent processing of prepilin in both cases (Fig. 3). Thus, GspO can cleave an authentic type IV pilin precursor.

Partial processing of gonococcal prepilin in E. coli K-12 is not due to GspO activity. We next tested whether a gspO (hopD)::Km insertion mutation (30) had any effect on the partial processing of gonococcal prepilin observed in E. coli K-12 at 37°C but not at 30°C (7, 12). As shown in Fig. 4, the level of prepilin processing in this strain was the same as that in the parent (gspO+) (Fig. 4). The presence of the gspO mutation was verified by PCR amplification of the gspO gene from the parent and mutant strains. With the parent, the only amplified product obtained was a 0.8-kb fragment, as expected from the positions of the primers at the ends of the gspO gene. With the mutant, however, the only amplified product was a 2.1-kb frag-

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TABLE 2. E. coli K-12 strains used in this study

| Strain       | Characteristics | Source or reference |
|--------------|-----------------|---------------------|
| MC4100       | F- araD139 Δ(araF-lac)169 rpsL150 relA1 hfbB5301 deoC1 pbfsF5 | Laboratory strain |
| PAP105       | Δ(lac-pro) F' lac proph lacZ15 pro+ | Laboratory strain |
| PAP7232      | Same as MC4100 but with pul gene cluster inserted into chromosome and carrying F' lac proph lacZ15 pro+ | 21, 23 |
| PAP7228      | Same as PAP7232 but ΔpulG3 | 21, 23 |
| PAP7245      | Same as PAP7232 but ΔpulO | 21, 23 |
| RRI          | ara-14 pro2 lacY1 galK2 resL-5 mtl-1 supF44 hsdS20 | 30 |
| RRI1HD       | Same as RRI but gspO (hopD)::Km | 30 |

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TABLE 3. Complementation of pulO and pulO mutations by cloned gspG and gspO genes

| Strain | Mutation | Plasmid | Gene | IPTG inductiona | Secretion (%)b |
|--------|----------|---------|------|----------------|----------------|
| PAP7232 | pulO     | −/+     | −/+  | 80–100         | 80–100         |
| PAP7245 | pulO     | −/+     | −/+  | 15–20          | 15–20          |
|         | gspO     | −       | +    | 85–90          | 85–90          |
|         | −       | −       | 85–90 |  | 85–90          |
| PAP7228 | pulO     | −/+     | −/+  | 45–50          | 45–50          |
|         | gspO     | −       | +    | 100            | 100            |
|         | −       | −       | <5   |  | <5            |

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a −/+ with or without IPTG production; −, without IPTG induction; +, with IPTG induction.

b Secretion levels are expressed as the ratio of the cell-associated pullulanase that is accessible to substrate (pullulan) in whole cells.
ment, consistent with the presence of the kanamycin resistance gene cartridge in gspO (data not shown). Therefore, we conclude that the partial processing of prePile is due to an enzyme other than GspO. One possible candidate is the product of another prepilin peptidase gene homolog recently discovered in the E. coli K-12 chromosome (18). Alternatively, gonococcal prepilin might be partially clipped by a nonspecific protease when it is produced in E. coli.

GspG can substitute for PulG in pullulanase secretion and is also processed by GspO prepilin peptidase. GspG is homologous to PulG (63% identical residues excluding the common, N-terminal hydrophobic domain), one of the type IV prepilin-like components of the pullulanase secretion system (21, 22, 24). Since at least one PulG homolog, the Erwinia chrysanthemi OutG protein (22), can substitute for PulG, we tested the ability of gspG to complement the pulG mutation in E. coli PAP7228 (Table 2). This strain normally releases <5% of its pullulanase onto the cell surface. However, transformants of this strain carrying pCHAP4010, a pUC18 derivative which carries the gspG gene under lac promoter control (see Materials and Methods), secreted considerably more of their pullulanase (24% of total enzyme activity) than when grown in the presence of IPTG (Table 2). The ability of induced gspG to complement a pulG mutation is in contrast to the situation for gspO (Table 3) and outG, which inhibit secretion at high levels (21, 22), probably through titration by their products of one or more of the other factors required for pullulanase secretion. Thus, it would appear that GspG can replace PulG in the pullulanase secretion machinery, but its affinity for this machinery may be lower than that of PulG so that it cannot titrate another essential component, or its level of production may be inadequate to cause transinhibition.

In agreement with the fact that GspG is quite similar to PulG and to its P. aeruginosa homolog XcpT; 46% residue identity to GspG excluding the hydrophobic region), a protein whose size corresponded to that of GspG (28) was readily detected with both PulG- and XcpT-specific antisera by immunoblotting of extracts of E. coli cells expressing gspG from the lacZ promoter. The PulG-specific antiserum reacted only weakly, however, and several endogenous E. coli proteins also reacted with this antiserum at the dilution used (data not shown). The XcpT-specific antiserum reacted strongly with GspG and did not react with any other E. coli protein. This antiserum was therefore used to examine pre-GspG processing by GspO. As shown in Fig. 5, pre-GspG was processed by both GspO and PulO. Almost complete processing by PulO was observed even when the pulO gene was not induced (presumably because of incomplete repression of the lacZ promoter by lacIq), but processing of preGspG by GspO was observed only when gspO was induced with IPTG. These data could indicate that pre-GspG is a better substrate for PulO than for GspO, but it is important to note that the levels of gspO and pulO expression might not be the same, even though the vector is the same in both cases. It will be noted that PulO was also apparently more active than GspO in comparable tests with pre-PulG and prePile as substrates (Fig. 2 and 3).

The chromosomal copies of gspG and gspO are poorly expressed. Both gspG and gspO were shown above to encode functional proteins, but E. coli K-12 strains in which the pul operons have been integrated into the chromosome are only able to secrete pullulanase long as the pulG and pulO genes are expressed (21, 23). This observation indicates that the chromosomal copies of neither gspG nor gspO are expressed sufficiently to complement pulG or pulO mutations, respectively. Furthermore, a protein corresponding to GspG could not be detected in extracts of E. coli K-12 strains examined by immunoblotting with XcpT-specific antiserum (data not shown).

These data imply that the gspG and gspO genes are cryptic, presumably because they are not transcribed. According to the DNA sequence data, the gspC-O operon does not contain intergenic regions of sufficient length to contain promoters (17) (Fig. 1). Thus, transcription of the entire operon, if it occurs, is likely to be initiated upstream from gspC, as is the case for pulC-O (5) and the E. chrysanthemi outC-outO operon (2). We therefore amplified the region of the E. coli chromosome corresponding to the presumed promoter region of the gspC-O operon and cloned it into the promoter probe vector pRS551 (27) to give pCHAP4053. As shown in Table 4, β-galactosidase activity, which reflects transcription of the gspC-lacZ operon fusion, was readily detectable in cells carrying the resulting plasmid (mean of 865 Miller units). For comparison, the malE promoter (26) cloned into the same vector gave a mean of 2,848 Miller units when the cells were grown in the presence of IPTG (28) and outO (5). The E. chrysanthemi outC-outO operon (2). We therefore amplified the region of the E. coli chromosome corresponding to the presumed promoter region of the gspC-O operon and cloned it into the promoter probe vector pRS551 (27) to give pCHAP4053. As shown in Table 4, β-galactosidase activity, which reflects transcription of the gspC-lacZ operon fusion, was readily detectable in cells carrying the resulting plasmid (mean of 865 Miller units). For comparison, the malE promoter (26) cloned into the same vector gave a mean of 2,848 Miller units when the cells were grown in the presence of IPTG. When the gspC-lacZ operon fusion was integrated into the chromosome by homologous recombination at the attB site to give strain MC4100 CHAP4053 (see Materials and Methods), the level of β-galactosidase activity was above background (mean of 25 Miller units [Table 4] compared with means of 122 and 836 Miller units for the uninduced and induced malK promoter [26], respectively) but the cells were Lac- on MacConkey lactose agar, on minimal lactose agar, and on L agar containing the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, indicating that the gspC promoter is only inducible.
The gspA gene, which is divergently transcribed from gspC (Fig. 1), is homologous to the A. hydrophila exoA gene, which codes for a GSP-MTB component that appears to be absent from the pullulanase secretion system (10). The region between the translation start sites of gspA and gspC is quite short (179 bp) (17), suggesting that the divergent promoters might be coordinately expressed. We therefore tested the level of expression of the gspA promoter in the same way as described above for the gspC promoter by inserting the amplified DNA in reverse orientation. As shown in Table 4, the level of expression of the gspA promoter is even lower than that of the gspC promoter and was barely above background level when integrated into the chromosome.

The gspC-gspA intergenic region does not contain sequences similar to the binding sites for known regulator proteins, including global regulators such as cyclic AMP receptor protein, but it does contain a nearly perfect, 25-bp A-T-rich inverted repeat (TATGGATTATTAAATGACTAAT) upstream from the only potential −35 region of gspC. This inverted repeat could be the binding site for a protein that controls gspC and/or gspA expression. The difference between the levels of β-galactosidase activities in cells carrying gspC-lacZ or gspA-lacZ operon fusions on plasmids or integrated into the chromosome (Table 4) is somewhat greater than the estimated pRS551 copy number (approximately 20). The higher-than-expected level of operon fusion expression in the pRS551 derivatives could be explained by titration of a hypothetical repressor that controls expression of the putative gspA-gspC promoter by binding to this inverted repeat. This possibility was tested by cloning this intergenic region into the high-copy-number plasmid pUC18 (to give pCHAP4051) and then introducing this plasmid into strains PAP7228 and PAP7245 (to look for complementation of the pulG and pulO mutations by derepressed chromosomal gspG and gspO, respectively) and into MC4100 CHAP4053 (to test for derepression of gspC-lacZ expression). None of these tests provided any evidence for titration of a repressor protein by pCHAP4051.

Concluding remarks. The results presented here show that two E. coli chromosomal genes previously identified and sequenced by others (gspG and gspO) (1, 17, 28, 30) code for components of a GSP but are expressed at such low levels during growth in LB broth that they are nonfunctional. This makes it highly unlikely that the MTB of the E. coli K-12 GSP is operational under standard laboratory conditions, in line with the generally accepted notion that this bacterium does not secrete extracellular proteins. This raises some important questions which we intend to investigate. (i) Are the E. coli gsp genes activated under particular growth conditions? (ii) Is E. coli then able to secrete one or more extracellular proteins, and if so, which proteins are secreted? (iii) Are the genes coding for these secreted proteins expressed under normal growth conditions, or is their expression linked to that of the gsp genes? (iv) Are the gsp genes under the control of a repressor or an activator protein? (v) Is expression of the gsp genes linked to that of the E. coli hop and pdp genes, which appear to code for components of a type IV pilus biogenesis pathway (30)? (vi) Are the gsp genes expressed in other E. coli strains in which they are present (28)?

The studies reported here provide some of the tools with which to answer these questions. For example, the chromosomal gspC-lacZ and gspA-lacZ operon fusions could be used to study the level of expression of the gsp genes under different growth conditions and in strains carrying mutations in potential regulator genes. Furthermore, these fusions, together with the ability of gspG and gspO to complement mutations in the pulG and pulO genes, respectively, provide means of selecting for mutations which increase the level of gsp gene expression.

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REFERENCES

1. Andrews, S. C., P. M. Harrison, and J. R. Guest. 1989. Cloning, sequencing, and mapping of the bacterioccin gene (bfr) of Escherichia coli K-12. J. Bacteriol. 171:3040–3047.
2. Condemine, G., C. Dorel, N. Hugouvieux-Cotte-Pattat, and J. Robert-Baudouy. 1992. Some of the out genes involved in the secretion of pectate lyases in Erwinia chrysanthemi are regulated by kdgR. Mol. Microbiol. 6:3199–3211.
3. d’Enfert, C., and A. P. Pugsley. 1989. Klebsiella pneumoniae pulG gene encodes an outer membrane lipoprotein required for pullulanase secretion. J. Bacteriol. 171:3673–3679.
4. d’Enfert, C., I. Reys, C. Wandesmar, and A. P. Pugsley. 1989. Protein secretion by Gram-negative bacteria: characterization of two membrane proteins required for pullulanase secretion by Escherichia coli K-12. J. Biol. Chem. 264:17462–17468.
5. d’Enfert, C., A. Ryter, and A. P. Pugsley. 1987. Cloning and expression in Escherichia coli of the Klebsiella pneumoniae pulG genes for production, surface localization and secretion of the lipoprotein pullulanase. EMBO J. 6:3531–3538.
6. Dupuy, B. 1994. Unpublished data.
7. Dupuy, B., and A. P. Pugsley. 1994. Type IV prepilin peptidase gene of Neisseria gonorrhoeae MS11: presence of a related gene in other piliated and nonpiliated Neisseria strains. J. Bacteriol. 176:1323–1331.
8. Hardie, K. R., S. Lory, and A. P. Pugsley. 1996. Insertion of an outer membrane protein in Escherichia coli requires a chaperone-like protein. EMBO J. 15:978–988.
9. Howard, S. P., J. Critch, and A. Bedi. 1993. Isolation and analysis of eight exe genes and their involvement in extracellular protein secretion and outer membrane assembly in Aeromonas hydrophila. J. Bacteriol. 175:6695–6703.
10. Jahagirdar, R., and S. P. Howard. 1994. Isolation and characterization of a second exe operon required for extracellular protein secretion in Aeromonas hydrophila. J. Bacteriol. 176:6819–6826.

### Table 4. β-Galactosidase activities of gsp-lacZ transcriptional fusions

| Strain       | Plasmid    | lac fusion | β-Galactosidase activity (Miller units)* |
|--------------|------------|------------|----------------------------------------|
| MC4100       | pRS551     | gspC-lacZ  | 8.8 ± 1.8                              |
| MC4100       | pCHAP4053  | gspA-lacZ  | 20.3 ± 24.6                            |
| MC4100 ARSS51| CHAP4048   | gspC-lacZ  | 0.8 ± 0.4                              |
| MC4100 CHAP4053 | pCHAP4048 | gspA-lacZ  | 25.0 ± 0.3                             |
| MC4100 CHAP4048 | pCHAP4048 | gspA-lacZ  | 2.0 ± 0.7                              |

* Cells were grown in LB broth at 37°C to early log phase, and β-galactosidase activity was determined according to the method of Miller (14). The 179-bp fragment from the gsp operon (GenBank accession no. U18997; coordinates 236147 to 236326) was cloned in both orientations into vector pRS551. Lambda phage derivatives of these plasmids were made and used to lysogenize MC4100 cells as described by Simons et al. (27).

Mean values and standard deviations are calculated from the data obtained in four independent measurements with the same strain.

Concluding remarks. The results presented here show that two E. coli chromosomal genes previously identified and sequenced by others (gspG and gspO) (1, 17, 28, 30) code for components of a GSP but are expressed at such low levels during growth in LB broth that they are nonfunctional. This
11. Jiang, B., and S. P. Howard. 1992. The Aeromonas hydrophila exeE gene, required both for protein secretion and normal outer membrane biogenesis, is a member of a general secretion pathway. Mol. Microbiol. 6:1351–1361.

12. Lauer, P., N. H. Albertson, and M. Koomey. 1993. Conservation of genes encoding components of a type IV pilus assembly/two-step protein export pathway in Neisseria gonorrhoeae. Mol. Microbiol. 8:357–368.

13. Michaelis, S., C. Chapon, C. d’Enfert, A. P. Pugsley, and M. Schwartz. 1985. Characterization and expression of the structural gene for pullulanase, a maltose-inducible secreted protein of Klebsiella pneumoniae. J. Bacteriol. 164:633–638.

14. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

15. Nunn, D., and S. Lory. 1991. Product of the Pseudomonas aeruginosa gene pilD is a prepilin leader peptidase. Proc. Natl. Acad. Sci. USA 88:3261–3285.

16. Nunn, D., and S. Lory. 1992. Components of the protein excretion apparatus of Pseudomonas aeruginosa are processed by the type IV prepilin peptidase. Proc. Natl. Acad. Sci. USA 89:47–51.

17. Ptunkett, G. 1995. Escherichia coli K-12 chromosomal region from 67.4 to 76.0 minutes. GenBank U18997. Locus ECOUW67.

18. Ptunkett, G. 1995. Escherichia coli K-12 genome; approximately 65 to 68 minutes. GenBank U28377. Locus ECU28377.

19. Possot, O. 1994. Unpublished data.

20. Pugsley, A. P. 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol Rev. 57:50–108.

21. Pugsley, A. P. 1993. Processing and methylation of PulG, a pilin-like component of the general secretory pathway of Klebsiella oxytoca. Mol. Microbiol. 9:295–308.

22. Pugsley, A. P. Multimers of the precursor of a type IV pilin-like component of the general secretory pathway are unrelated to pili. Mol. Microbiol., in press.

23. Pugsley, A. P., and B. Dupuy. 1992. An enzyme with type IV prepilin peptidase activity is required to process a component of the general extracellular protein secretion pathway of Klebsiella oxytoca. Mol. Microbiol. 6:751–760.

24. Pugsley, A. P., and O. Possot. 1993. The general secretory pathway of Klebsiella oxytoca: no evidence for relocalization or assembly of pilin-like PulG protein into a multiprotein complex. Mol. Microbiol. 10:665–674.

25. Pugsley, A. P., and J. Reys. 1990. Five genes at the 3’ end of the Klebsiella pneumoniae pulC operon are required for pullulanase secretion. Mol. Microbiol. 4:365–379.

26. Plunkett, G. 1995. Escherichia coli K-12 chromosomal region from 67.4 to 76.0 minutes. GenBank U18997. Locus ECOUW67.

27. Plunkett, G. 1995. Escherichia coli K-12 genome; approximately 65 to 68 minutes. GenBank U28377. Locus ECU28377.

28. Stojiljkovic, I., R. Schönherr, and J. G. Kusters. 1995. Identification of the hopG gene, a component of Escherichia coli K-12 type II export system, and its conservation among different pathogenic Escherichia coli and Shigella isolates. J. Bacteriol. 177:1892–1895.

29. Takeshita, S., M. Sato, M. Toba, W. Masahashi, and T. Hashmoto-Goto. 1987. High copy number and low copy number plasmid vectors for lacZ a-complementation and chloramphenicol- or kanamycin-resistance selection. Gene 61:53–74.

30. Whitchurch, C. B., and J. S. Mattick. 1994. Escherichia coli contains a set of genes homologous to those involved in protein secretion, DNA uptake and the assembly of type-4 fimbriae in other bacteria. Gene 150:9–15.