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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 *gspC-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 pullulanase 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, 15) 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 *pulG* 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.*

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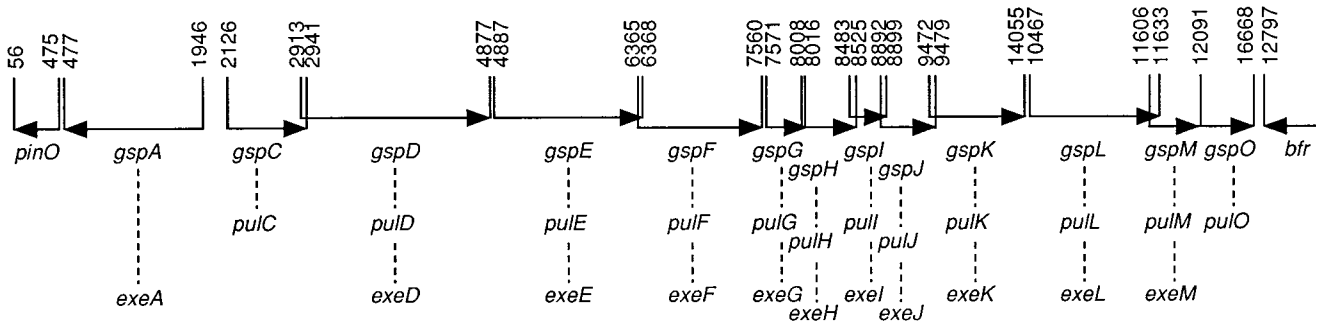

FIG. 1. Organization of *gsp* and flanking genes in the min 73 region of the *E. coli* K-12 chromosome as determined by sequence analysis (17). Overlapping genes are shown on different lines. Numbers indicate the positions of the first and last codons of each gene relative to an arbitrary coordinate upstream from the *pinO* gene. The *gspA-gspC* intergenic region is presumed to include the promoters for the putative *gspC-O* and *gspA-pinO* operons. The *K. oxytoca* (*pul*) and *A. hydrophila* (*exe*) genes which are homologous to the *gsp* genes are also indicated. The representation is not meant to indicate that the gene organization is the same in all three cases: *K. oxytoca* and *A. hydrophila* have an additional gene (*pulN* and *exeN*, respectively) located downstream from *pulM* and *exeM* (9, 25), the *A. hydrophila* gene coding for a prepilin peptidase (corresponding to *gspO* and *pulO*) is not located in the same region of the chromosome as the *exeD-N* operon (9), the *A. hydrophila* homolog of the *gspC* gene has not been identified (11), and *K. oxytoca* is not known to have a homolog of the *gspA* or *exeA* gene (5).

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 prepilin peptidase (7, 12). However, another potential substrate for the GspO prepilin 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 prePilE 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 (*gspF*) or within the 5′ end of the following gene (*gspH*). The primers introduced a unique *Eco*RI site at the 5′ end of the amplified fragment and a unique *Hin*dIII site at its 3′ end. The amplified DNA was cleaved with *Eco*RI and *Hin*dIII and cloned into the corresponding sites of pUC18 to give pCHAP4010 (Table 1). The insert in pCHAP 4043 (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 *Bam*HI sites at both ends of the amplified fragment, which was cloned into the *Bam*HI 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-β-D-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-

TABLE 1. Plasmids used in this study[a]

Plasmid	Characteristics	Source or reference
pBSHopD	pBluescript carrying *gspO* (*hopD*) from pGS280 (1) under *lacZp* control (Ap^r)	30
pCHAP4015	pHSG576 (28) carrying *gspO* cloned from pBSHopD as an *Xho*I-*Bam*HI fragment under *lacZp* control (Cm^r)	This study
pCHAP155	pHSG575 carrying *pulO* under *lacZp* control (Cm^r)	23
pCHAP158	$pEMBL8^-$ carrying *pulO* under *lacZp* control (Ap^r)	23
pCHAP4010	pUC18 carrying PCR-amplified *gspG* gene under *lacZp* control (Ap^r)	This study
pCHAP4043	pACYC184 carrying PCR-amplified *gspG* gene cloned under control of the constitutive *cat* promoter (Tc^r)	This study
pCHAP162	$pBGS19^+$ carrying *pulG* under *lacZp* control	23
pNG1100	pBR322 carrying *pilE* cloned from *N. gonorrhoeae* MS11 (Ap^r)	M.-K. Taha
pNG300	pGB2 carrying *pilE* cloned from *N. gonorrhoeae* MS11 (Cm^r)	7
pRS551	*lacZ* operon fusion promoter probe vector (Km^r)	27
pCHAP4053	pRS551 carrying putative *gspA-gspC* promoter region cloned as a *Bam*HI fragment to create a *gspC-lacZ* operon fusion (Ap^r)	This study
pCHAP4048	Same as pCHAP4053 but with fragment in reverse orientation to create a *gspA-lacZ* operon fusion (Ap^r)	This study
pCHAP4051	pUC18 carrying same *Bam*HI fragment as pCHAP4053 (Ap^r)	This study

[a] Further details of plasmids constructed as part of the present study are given in Materials and Methods.

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 flbB5301 deoC1 ptsF25*	Laboratory strain
PAP105	Δ(*lac-pro*) F′ *lacI*^{q} Δ*lacZM15 pro*$^+$ Tn*10*	Laboratory strain
PAP7232	Same as MC4100 but with *pul* gene cluster inserted into chromosome and carrying F′ *lacI*^{q} Δ*lacZM15 pro*$^+$ Tn*10*	21, 23
PAP7228	Same as PAP7232 but Δ*pulG3*	21, 23
PAP7245	Same as PAP7232 but Δ*pulO*	21, 23
RRI	*ara-14 proA2 lacY1 galK2 resL xyl-5 mtl-1 supE44 hsdS20*	30
RR1HD	Same as RR1 but *gspO* (*hopD*)::Km	30

merly 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-indolyl phosphate, 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). β-Galactosidase was assayed according to the method of Miller (14).

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 prePulG 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*.

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

Strain	Mutation	Plasmid	Gene	IPTG induction[a]	Secretion (%)[b]
PAP7232				−/+	80–100
PAP7245	*pulO*			−/+	<5
		pBSHopD	*gspO*	−	15–20
				+	85–95
		pCHAP158	*pulO*	−	85–90
				+	85–90
PAP7228	*pulG*			−/+	<5
		pCHAP4010	*gspG*	−	45–50
				+	100
		pCHAP162	*pulG*	−	80–100
				+	<5

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

[b] Secretion levels are expressed as the relative amount of the cell-associated pullulanase that is accessible to substrate (pullulan) in whole cells.

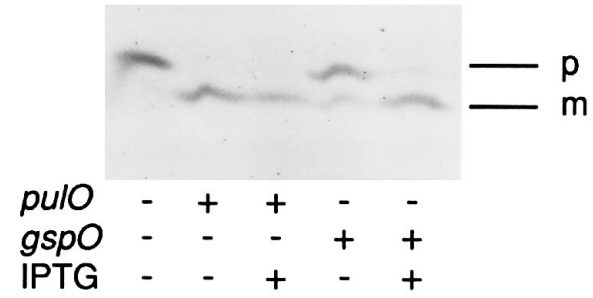

FIG. 2. Processing of prePulG in cells expressing *pulO* or *gspO*. Cell extracts from strain PAP7245 (Δ*pulO*), PAP7245(pCHAP158) (*pulO*$^+$), or PAP7245 (pBSHopD) (*gspO*$^+$) grown in medium containing 0.4% maltose with or without 1 mM IPTG (as indicated) were tested by immunoblotting with PulG-specific antiserum. p, prePulG; m, mature PulG. Only the region of the immunoblot displaying PulG is shown.

Similar tests were performed to determine whether the *gspO* gene product could cleave *N. gonorrhoeae* prePilE. 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 prePilE in both cases (Fig. 3). Thus, GspO can cleave an authentic type IV pilin precursor.

Partial processing of gonococcal prePilE 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 prePilE 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*$^+$). 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-

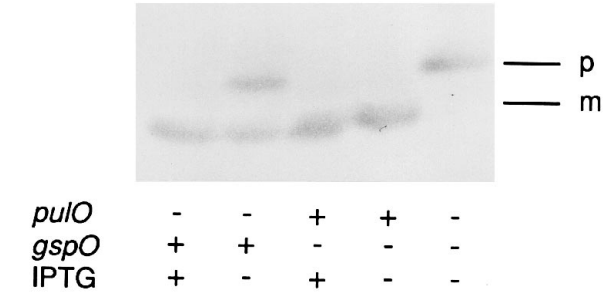

FIG. 3. Processing of *N. gonorrhoeae* prepilin in cells expressing *gspO* or *pulO*. Extracts of cells carrying pNG300 together, where indicated, with pBSHopD (*gspO*$^+$) or pCHAP158 (*pulO*$^+$) were examined by immunoblotting with pilin-specific antiserum. Cells were grown in medium with or without IPTG as indicated. p, prepilin; m, mature pilin. Only the region of the immunoblot displaying pilin is shown.

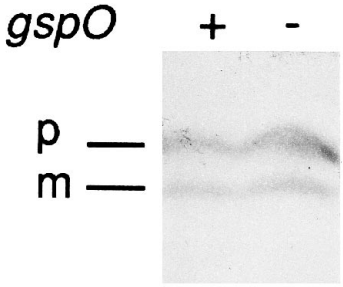

FIG. 4. Processing of *N. gonorrhoeae* prepilin in *E. coli* devoid of exogenous prepilin peptidase genes. Extracts of cells of strains RR1 (*gspO*$^+$) and RR1 *gspO*::Km (*gspO* mutant), each carrying NG1100, were examined by immunoblotting with pilin-specific antiserum. p, prepilin; m, mature pilin. Only the region of the immunoblot displaying pilin is shown.

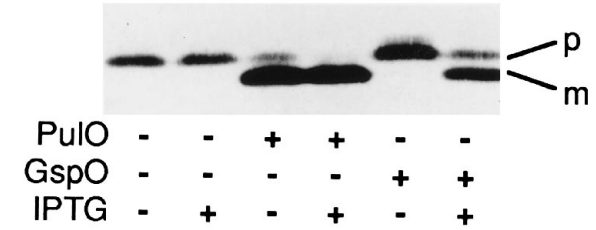

FIG. 5. Processing of preGspG in *E. coli* K-12 MC4100 F′ *lacI*^{q} producing PulO or GspO. Extracts of cells carrying pCHAP4043 either alone or in combination with pCHAP155 (*pulO*$^+$) or pCHAP4015 (*gspO*$^+$) and grown in the absence or presence of IPTG as indicated were examined by immunoblotting with XcpT-specific antiserum. p, preGspG; m, mature GspG. Only the region of the immunoblot displaying GspG is shown.

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 must be 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 when grown in the absence of IPTG (uninduced *gspG* expression) and were 100% secretion proficient when grown in the presence of IPTG (Table 3). The ability of induced *gspG* to complement a *pulG* mutation is in contrast to the situation for *pulG* (Table 3) and *outG*, which inhibit secretion when expressed 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 XcpG (formerly 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 XcpG-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 XcpG-specific antiserum reacted strongly with GspG and did not react with any other *E. coli* protein. This antiserum was therefore used to examine preGspG processing by GspO. As shown in Fig. 5, preGspG 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 *lacI*^{q}), but processing of preGspG by GspO was observed only when *gspO* was induced with IPTG. These data could indicate that preGspG 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 prePulG 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* genes have been integrated into the chromosome are only able to secrete pullulanase as 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 the XcpG-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 absence of the inducer maltose, and β-galactosidase activity was increased approximately eight times when the cells were grown in the presence of the inducer maltose. When the *gspC-lacZ* operon fusion was integrated into the chromosome by homologous recombination at the *att*λ site to give strain MC4100 λCHAP4053 (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

TABLE 4. β-Galactosidase activities of *gsp-lacZ* transcriptional fusions[a]

Strain	Plasmid	*lac* fusion	β-Galactosidase activity (Miller units)[b]
MC4100	pRS551		8.8 ± 1.8
MC4100	pCHAP4053	*gspC-lacZ*	864.6 ± 14.4
MC4100	pCHAP4048	*gspA-lacZ*	102.3 ± 24.6
MC4100 λRS551			0.8 ± 0.4
MC4100 λCHAP4053		*gspC-lacZ*	25.0 ± 0.3
MC4100 λCHAP4048		*gspA-lacZ*	2.0 ± 0.7

[a] 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).

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

weakly active. This low level of *gspC* promoter activity presumably explains why *gspG* and *gspO* are expressed very poorly (if at all).

The *gspA* gene, which is divergently transcribed from *gspC* (Fig. 1), is homologous to the *A. hydrophila exeA* 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 (TATGTATTTAATTAATAAATACATA) 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 λCHAP4053 (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 *ppd* 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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