

Biochemistry and Regulation of a Novel *Escherichia coli* K-12 Porin Protein, OmpG, Which Produces Unusually Large Channels

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A novel porin, OmpG, is produced in response to a chromosomal mutation termed *cog-192*. Molecular characterization of *cog-192* revealed that it is a large chromosomal deletion extending from the 3' end of *pspA* through to the 5' end of an open reading frame located immediately upstream of *ompG*. As a result of this 13.1-kb deletion, the expression of *ompG* was placed under the control of the *pspA* promoter. Characterization of OmpG revealed that it is quite different from other porins. Proteoliposome swelling assays showed that OmpG channels were much larger than those of the OmpF and OmpC porins, with an estimated limited diameter of about 2 nm. The channel lacked any obvious solute specificity. The folding model of OmpG suggests that it is the first 16-stranded β -barrel porin that lacks the large external loop, L3, which constricts the channels of other nonspecific and specific porins. Consistent with the folding model, circular dichroism showed that OmpG contains largely a β -sheet structure. In contrast to other *Escherichia coli* porins, there is no evidence that OmpG exists as stable oligomers. Although *ompG* DNA was present in all *E. coli* strains examined so far, its expression under laboratory conditions was seen only due to rare chromosomal mutations. Curiously, OmpG was constitutively expressed, albeit at low levels, in *Salmonella*, *Shigella*, and *Pseudomonas* species.

The outer membrane of gram-negative bacteria provides a barrier against noxious agents in the environment. *Escherichia coli* K-12 contains a set of outer membrane porin proteins that form channels allowing an influx of nutrients (for reviews, see references 3, 21, and 25). Two porins, OmpF and OmpC, often regarded as the classical porins, allow the flow of water-soluble solutes with molecular weights of around 500 or less (20, 25). Although these general pores lack any pronounced solute specificity, they are weakly cation selective. The PhoE porin is structurally similar to the classical porins and forms general diffusion pores with a preference for anionic solutes (26). Other porins can specifically facilitate the uptake of substrates such as maltodextrins (LamB [40]) and nucleosides (Tsx [15]). OmpA was initially thought to play only a structural role, but recent *in vitro* studies have shown that this protein also displays weak channel activity (37). Similarly, TolC has been shown to form channels *in vitro* (3).

In the last decade, publications pertaining to structure-function analysis of the porins provided a better understanding of their molecular anatomy. A major breakthrough came with the three-dimensional structural resolutions of OmpF (6) and PhoE (6, 10). The monomeric subunit of each of these homotrimeric porins consists of 16-stranded antiparallel β barrels enclosing the transmembrane pore. The pore entrance is narrowed by long external loops, and the middle of the channel is constricted by the inward folding of loop L3, while its cross section increases abruptly just after this constriction zone (facing the periplasm). Proof that the constricted area defines the filtering ability of the channels came from genetic studies (2, 16, 17).

The classical porin proteins exist as trimers that display unusually stable structural properties (28). They form trimers that resist denaturation by strong anionic detergents such as sodium dodecyl sulfate (SDS). This high stability results from hydrophobic and electrostatic interactions between the subunits (6). Porin proteins do not contain long stretches of hydrophobic residues; instead, every alternate residue facing the lipid bilayer is hydrophobic. The presence of negatively charged residues on the outer surface allows these exposed surfaces to interact with the negatively charged groups of lipopolysaccharide via divalent cation bridges (25).

Porin proteins are critical for bacterial growth. Porin-deficient strains grow slowly and accumulate mutations that permit expression of new porin proteins. This finding led to the isolation of *E. coli* strains that acquired the ability to synthesize a new porin protein, NmpC (4). The origin of NmpC was linked to a defective prophage, QSR. Similarly, another porin, Lc, encoded by a lambdaoid bacteriophage, was isolated (8). The OmpG porin was discovered among mutants that gained increased outer membrane permeability in the absence of OmpF and LamB (18). However, unlike NmpC and Lc, OmpG is not encoded by a prophage genome. Normally LamB is required for transport of maltodextrins, sugars that are excluded from OmpF and OmpC channels because of their large size. Thus, a LamB⁻ OmpF⁺ OmpC⁺ strain displays a maltodextrin-minus (Dex⁻) phenotype. Strains with this composition revert to Dex⁺ at a low frequency (10⁻⁹ to 10⁻¹⁰). The mutations were mapped to four loci: *ompF* (2), *ompC* (16, 17), *imp* (30), and *cog* (18). *cog* (for control of *ompG*) mutations resulted in the appearance of a new outer membrane protein, OmpG. Growth and uptake experiments showed that OmpG displays a porin-like activity. Genetic experiments indicated that *cog* itself may code for a negative regulator of OmpG expression (18).

In this report, we provide data showing that OmpG defines

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a novel class of porin protein whose sequence, biochemical properties, and channel properties are distinct from those of known bacterial porins.

MATERIALS AND METHODS

Media. Luria broth and MacConkey media (both from Gibco) were prepared as described previously (33). The amount of maltodextrins (Pfsanstiehl) added to the MacConkey medium differed between preparations of the maltodextrin stock solution. The stock solution of maltodextrins was titrated by determining the amount of maltodextrins needed to yield red colonies for an OmpG⁺ strain (RAM123) and white colonies for an OmpG⁻ strain (DME553).

Bacterial strains and lambda phages from Kohara library. The laboratory *E. coli* K-12 strains used in this study were derived from DME553 (Δ *amb106* Δ *ompF80*). RAM123 is a Dex⁺ derivative of DME553 that produces OmpG due to the *cog-192* mutation (18). RAM194 was derived from RAM123 by transducing the Δ *ompC178* allele through a linked Tn10, thus making the resulting strain OmpC⁻. HS3169 (*malK::Tn10*) was obtained from Howard Shuman. Strains K1342 [*lps3.2*; Φ (*pspA'-lacZ*)] and K1472 (Δ *pspABC::kan*) were kindly provided by Peter Model. All other *E. coli* strains were obtained from *E. coli* Genetic Stock Center. *Salmonella typhimurium* LT2 (*ompD*⁺) and CH338 (*ompD*) were kindly provided by Ken Sanderson. *Shigella flexneri* serotype 2a (ATCC 9473) was obtained from the American Type Culture Collection. *Pseudomonas aeruginosa* PAO1 was from our laboratory strain collection. Clinical isolates of *Salmonella* strains were made available to us by Micah Williams. Lambda clones 250 through 260 from Kohara phage library (12) and *E. coli* LE392 for their propagation were kindly provided by Frederick Blattner.

Protein methods. Cell envelopes were prepared by the French press lysis method as described previously (19). Proteins were analyzed by electrophoresis through SDS-polyacrylamide (11%) gels (SDS-PAGE) (14). The following methods were used for OmpG purification. When OmpG was needed for raising antibodies or protein sequencing, envelopes obtained from a strain lacking OmpA, OmpC, OmpF, and LamB but producing OmpG from a plasmid were treated with 0.5% SDS in 50 mM Tris-HCl (pH 8.0) for 1 h at 37°C. SDS-solubilized proteins were removed by centrifugation at 18,000 × g for 30 min. The pellet was resuspended in the SDS sample buffer (2% SDS, 10% glycerol, 5% β -mercaptoethanol, 50 mM Tris-HCl [pH 6.8]) and solubilized by boiling for 5 min. Solubilized samples were analyzed by SDS-PAGE, and the gel was stained with Coomassie blue. The OmpG band was excised and electroeluted in a Bio-Rad electroeluter. Eluted OmpG samples were concentrated by ethanol precipitation at -20°C. The pellet was resuspended in 20 mM HEPES (pH 7.4) or double-distilled H₂O for protein sequence determination.

For proteoliposome swelling assays, OmpG was partially purified from envelopes by a two-step detergent solubilization method. Envelopes lacking all major outer membrane proteins except OmpG were resuspended in 0.5% *n*-octylglucoside (OG)-10 mM HEPES (pH 7.4) and incubated for 30 min at 37°C. The pellet recovered by centrifuging samples at 436,000 × g for 30 min was resuspended in 1.0% OG-10 mM HEPES (pH 7.4). After incubation for 30 min at 37°C, samples were centrifuged as described above, and the supernatant containing primarily OmpG was recovered. Extract containing 0.2 μ g of protein was used for reconstitution of proteoliposomes as described previously (13) except that (i) a 30:2 mixture of acetone-washed phosphatidylcholine (Avanti Polar Lipids) and dicytolphosphate (Sigma) was used instead of *E. coli* phospholipids and (ii) dextran T-40 (Pharmacia) was used instead of dextran T-20. Proteoliposomes containing the dextran were diluted into iso-osmotic solutions of various solutes, and the permeation rates were measured by the initial rates of decrease of optical density of proteoliposome suspension (13).

OmpG for circular dichroism measurement was purified as follows. The outer membrane fraction of RAM194 was isolated by sucrose density centrifugation and extracted with 2% octyl-polyoxyethylene (Alexis Biochemicals). The extract was applied to a Bio-Rad DEAE-5PW high-pressure liquid chromatography column, which was eluted with a 0 to 0.5 M linear gradient of NaCl in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA-0.2% Lubrol PX. The fraction containing essentially pure OmpG was dialyzed against the buffer just mentioned but without NaCl, and its spectrum was measured, after concentration by ultrafiltration with Amicon PM-10 membrane, with an Aviv model 62DS circular dichroism spectrometer.

Antibodies were raised as described previously (19). For protein sequence analysis, electroeluted OmpG in double-distilled H₂O was acidified with trichloroacetic acid and dissolved with acetonitrile. Approximately 100 pmol of OmpG protein was spotted on a fiberglass disk (Beckman) and sequenced in a Proton 2090 protein sequencer (Beckman). For Western blot analysis, protein samples analyzed by PAGE were transferred to Immuno-Lite membranes (Bio-Rad), using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The Western blot analysis was carried out with solutions obtained from Bio-Rad and a 1:5,000 dilution of the OmpG antiserum.

DNA and RNA methods. *ompG*⁺ plasmid clones were obtained by transforming an OmpG⁻ strain with a *Sau3A* chromosomal gene bank, prepared from an *ompG*⁺ strain, and selecting for Dex⁺ (red) colonies on MacConkey-maltodextrin-ampicillin (50 μ g/ml) medium. Confirmation that the Dex⁺ phenotype was conferred by the expression of OmpG from a plasmid was obtained by retrans-

forming the plasmid into an OmpG⁻ strain and observing the Dex phenotype of transformants. *cog*⁺ plasmid clones were also obtained from a *Sau3A* chromosomal gene bank. The DNA sequence of cloned DNA was determined by the dideoxy-chain termination method (31). Sequence analysis was carried out with software packages from Genetics Computer Group Inc. (University of Wisconsin, Madison) and DNAMAN (Lynnon BioSoft). Lambda phages were purified through CsCl isopycnic gradients, and DNA was extracted as described previously (29). Chromosomal DNA and total RNA were isolated by a kit obtained from Amersham Life Science. Restriction enzyme digestions were carried out as instructed by the manufacturer. Southern and Northern analyses were carried out by standard protocols (29). Nucleic acids were cross-linked to Quantum Yield filters (Promega) in a Stratilinker (Stratagene). Nonradioactive hybridization probes were prepared by using a random-primer fluorescein labeling kit (Du Pont-NEN).

Nucleotide sequence accession number. Nucleotide sequences of the *ompG* gene and an upstream open reading frame (*orf1*) were assigned GenBank accession no. ECU49400.

RESULTS

Genetic mapping and cloning of *ompG*. Genetic mapping of *ompG* was facilitated by the isolation of linked transposon insertions. To obtain such insertions, a transposon (Tn10) insertion pool was prepared in a wild-type strain. The Tc^r (from Tn10) marker from this pool was transduced into an *ompG*⁺ (Dex⁺ Cog⁻) strain so as to obtain insertion mutations that prevented OmpG expression (Dex⁻ phenotype). These Tn10s were genetically mapped and found to be located at the 29-min region of the chromosome where *cog* had been previously mapped (18). Once *ompG* sequencing was completed (see below), *ompG*-specific primers were used to confirm the genetic location of *ompG* by using Kohara clones encompassing the 29-min region of the chromosome (12). Of the several Kohara clones used, two covering min 29.5 to 29.7 of the *E. coli* chromosome yielded *ompG*-specific fragments. Also, Southern blot analysis of Kohara clones with an *ompG*-specific probe confirmed its genetic location in the chromosome (data not shown).

The Tn10 insertion mutations resulting in a Dex⁻ (OmpG⁻) phenotype were found to be recessive, which allowed procurement of an *ompG*⁺ (Dex⁺) clone by complementation using an *E. coli* gene library. The original *ompG*⁺ plasmid clone contained a 5.5-kb chromosomal DNA insert. Subsequent subcloning showed that a 2.6-kb DNA fragment contains sufficient information for OmpG expression. The expression of OmpG from these plasmid clones was not repressible by either chromosomal or plasmid-borne Cog (data not shown). Gene dosage experiments showed that the lack of repression was not due to a possible titration of the repressor by an excessive number of operator sites. A reorientation of the *ompG*⁺ DNA insert with respect to the plasmid-borne Tc^r gene promoter resulted in no detectable OmpG expression, showing that the *ompG*⁺ clone lacked promoter sequences and possibly the site needed for Cog-mediated repression.

Nucleotide sequence analysis of the *ompG* region. The nucleotide sequence of the entire 2.6 kb of insert DNA was determined and found to match perfectly the published sequence (5). Two ORFs (*orf1* and *orf2*) within the *ompG*⁺ DNA insert were identified; *orf2* corresponded to OmpG. *orf2* starts 157 bp downstream from the end of *orf1* and encodes a 301-residue-long protein. The amino-terminal end of Orf2 contains a typical signal sequence motif of 21 amino acid residues. A 14-amino-acid NH₂-terminal sequence of the purified OmpG was identical to residues 22 to 35 deduced from DNA sequence of *orf2*, confirming that OmpG is coded by *orf2*.

OmpG is a novel porin protein. At the amino acid sequence level, OmpG shows no significant homology with other proteins in the data bank. Direct alignment of the OmpG sequence with the OmpF and OmpC sequences displayed a low

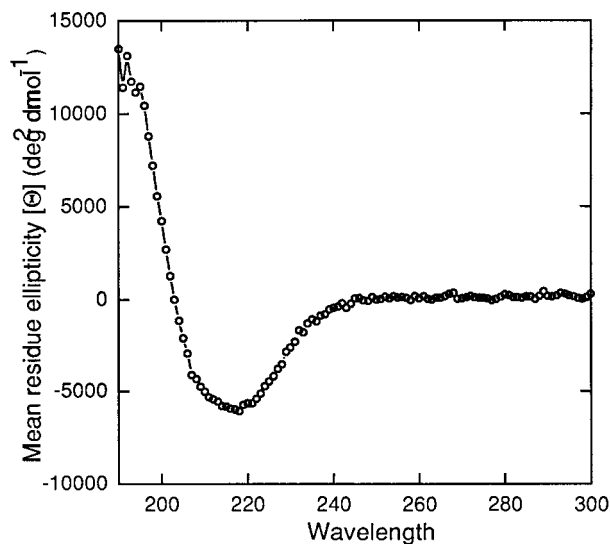


FIG. 1. Circular dichroism analysis of purified OmpG. Purification and measurement were carried out as described in Materials and Methods. The magnitude of mean residue ellipticity (about $-5,000 \text{ deg}^2 \text{ dmol}^{-1}$) is significantly smaller than that found with OmpA or *P. aeruginosa* OprF ($-9,000$ to $-10,000 \text{ deg}^2 \text{ dmol}^{-1}$ [38]), two outer membrane proteins that contain substantial α -helical domains. The spectrum also does not show the two sharp negative peaks at 208 and 220 nm, which are characteristic of α helices.

overall random identities of 22% (16 alignment gaps) and 16% (13 alignment gaps), respectively. That these identities are only superficial became apparent when we compared the primary sequence of OmpA with those of structurally and functionally unrelated proteins, OmpF and OmpC. Here again, the overall random identities were 22% (17 alignment gaps) between OmpA and OmpF and 17% (11 alignment gaps) between OmpA and OmpC. Finally, a low overall random identity of 17% (10 alignment gaps) was observed between OmpG and OmpA. On the other hand, between the two related proteins OmpF and OmpC, a striking 64% overall identity was observed with only seven alignment gaps.

Immunoblot analysis of filters containing purified *E. coli* OmpG, OmpC, OmpF, and LamB proteins showed no cross-reactivity of polyclonal OmpG antibodies with these proteins (data not shown). Furthermore, blotting of envelopes containing OmpA also gave negative signals. As expected, antibodies reacted strongly with either the purified OmpG protein or OmpG present in envelopes. Thus, OmpG is novel, as it shares neither sequence similarity nor antigenic overlap with the previously characterized outer membrane proteins of *E. coli*.

In certain respects, however, OmpG is related to other outer membrane proteins of *E. coli*. (i) Apart from its signal sequence, OmpG lacks any long hydrophobic stretches. (ii) OmpG, like some other porin proteins, lacks cysteine residues in its mature portion. (iii) The last residue of OmpG is phenylalanine, which is thought to be important for the proper insertion or assembly of outer membrane proteins (36). (iv) Like many outer membrane proteins, OmpG contains a preponderance of negatively charged residues (50 Asp and Glu, compared to 22 Arg and Lys), with an estimated pI of 4.4.

Circular dichroism and folding patterns of OmpG. To gain a better understanding of the secondary structure of OmpG, detergent-solubilized OmpG retaining its full channel activity was analyzed by circular dichroism (Fig. 1). The ellipticity pattern showed a minimum of a fairly small magnitude, close to 210 nm. This showed that in contrast to OmpA (38), there

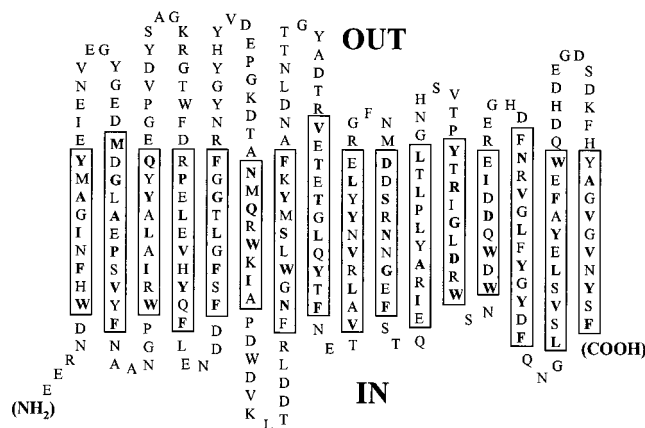


FIG. 2. Predicted folding pattern of OmpG. The OmpG amino acid sequence was analyzed by using computer programs based on the algorithms of Schirmer and Cowan (32) and Paul and Rosenbusch (27). Transmembrane β strands were predicted whenever a high value of hydrophobicity on alternate residues in a 10-residue stretch was found, and this prediction was strengthened by the presence of turn-promoting sequences on either side. Most of the transmembrane segments could be assigned unequivocally, but the presence of the 12th and 13th segments is less convincing, as the hydrophobicity of the alternate residues is not high.

was little contribution from α -helical structures and that OmpG had a largely β -sheet structure, similar to the classical porins. Based on the folding prediction methods of Schirmer and Cowan (predicts transmembrane stretches [32]) and Paul and Rosenbusch (predicts turns [27]), the mature portion of OmpG is predicted to have 16 transmembrane segments (Fig. 2, boxed regions). The amino acids at alternate positions within transmembrane segments are primarily hydrophobic residues that are predicted to have their side chains oriented outward toward the lipid environment. In general, the internal loops are smaller than those present on the outside, where there is a distinctive absence of an especially large loop, L3, a hallmark of the classical porins, where such a loop is shown to narrow the middle of the channel (6). The predicted OmpG folding model can now be tested by genetic and molecular means.

OmpG is a heat-modifiable protein. We have previously reported that unlike OmpF and OmpC, OmpG is not strongly associated within the cell envelope, as it can be completely solubilized with 1% SDS or Triton X-100 in the absence of high salt concentrations (18), a condition that does not allow solubilization of the classical porins. However, under milder solubilization conditions, such as treatment with 0.5% SDS or OG, the majority of OmpG remained associated with the membrane (Fig. 3). Interestingly, under these solubilization conditions when envelopes were analyzed by SDS-PAGE without heating, OmpG did not migrate at its denatured position of 34,000 mol wt but instead appeared as a faster-migrating species of 28,000 mol wt (Fig. 3, lane 7). Immunoblot analysis confirmed that this faster-migrating polypeptide corresponds to OmpG (data not shown). The treatment of envelopes with 1% OG led to the complete solubilization of OmpG without altering its heat-modifiable property. However, subsequent removal of OG by dialysis led to loss of this property (Fig. 3, lane 8). As shown below, both dialyzed and undialyzed preparations of OmpG were competent in forming active channels in proteoliposome swelling assays. The dialysis therefore appears to make the OmpG more susceptible to complete denaturation by SDS, presumably by removing stabilizing factors.

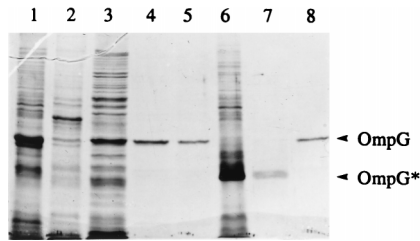


FIG. 3. SDS-PAGE analysis of envelopes and detergent-solubilized OmpG. Lane 1, untreated envelopes obtained from a strain lacking OmpF, OmpC, LamB, and OmpA; lane 2, pellet after OG extraction; lane 3, supernatant of 0.5% OG extraction; lane 4, supernatant of 0.5 and 1% OG extraction; lane 5, same as lane 4 but dialyzed against 10 mM HEPES (pH 7.4) buffer; lane 6, unheated envelopes; lane 7, same as lane 4 but unheated; lane 8, same as lane 5 but unheated. Positions of denatured OmpG and heat-modifiable OmpG (OmpG*) are shown. Samples present in lanes 1 to 5 were heated by boiling for 5 min prior to SDS-PAGE analysis.

In vitro channel properties of OmpG. In vivo growth and uptake experiments showed that OmpG has a porin-like activity that allows the influx of larger solutes than do the classical porins (18). Proteoliposome swelling assays (22, 23) were carried out to confirm the in vivo data.

Proteoliposomes were reconstituted from phosphatidylcholine and unfractionated outer membrane fragments from a strain lacking OmpF, OmpC, and LamB but containing OmpG. The influx of sugars was measured by the osmotic swelling of these vesicles. Several conclusions can be made from these experiments (Fig. 4). (i) OmpG produces fairly efficient channels. The rate of influx for a pentose, L-arabinose, was similar to that seen for proteoliposomes reconstituted with similar amounts of OmpC. Thus, at least for sugars, OmpG functions as well as the classical trimeric porins of *E. coli*. (ii) OmpG channels behaved strikingly differently from OmpF and OmpC when disaccharides were used. OmpF and OmpC

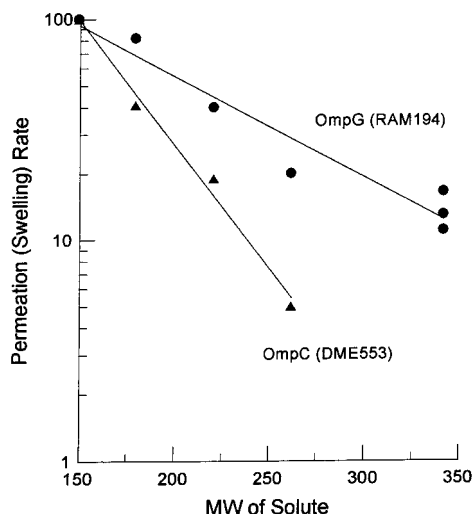


FIG. 4. Rates of penetration of various sugars through the OmpG channel. Proteoliposomes containing OmpG extract were made as described in Material and Methods, and portions of the suspension were diluted into iso-osmotic solutions of sugars to determine the rates of osmotic swelling of the vesicles. The rates were normalized to the swelling rate in L-arabinose, which was taken as 100%. The sugars used (and, in parentheses, their molecular weights) were L-arabinose (150), D-glucose (180), N-acetylglucosamine (221), 2,3-diacetamido-2,3-dideoxy-D-glucose (262), lactose (342), sucrose (342), and maltose (342). As a control, proteoliposomes containing OmpC extract were used.

showed disaccharide diffusion rates around 1 to 2% of the rate of L-arabinose diffusion (also see reference 24). In contrast, OmpG channels behaved as though they were much larger, allowing the diffusion of disaccharides at rates corresponding to 15 to 30% of the rates of L-arabinose. This behavior is very similar to that of the *P. aeruginosa* porin OprF, where a limiting pore diameter of 2.0 nm has been calculated (42). OprF, however, is different from OmpG in its low efficiency. Isolated OmpG (purified by solubilization in OG and fractionated by ion-exchange chromatography) and OmpF preparations were also used in proteoliposome swelling assays. The results validated the above finding, showing that OmpG channels were functionally larger than those of OmpF when a disaccharide (sucrose) and a trisaccharide (raffinose) were used. Calculations by the approach of Nikaïdo and Rosenberg (22) show that the dependence of permeability on solute size fits best with a pore diameter of about 20 Å. Since the distance between neighboring β strands is 4.5 Å, a β barrel with 16 transmembrane strands will have a diameter of 23 Å, measured between α -carbon atoms. With the amino acid side chains protruding into the interior of this barrel, the 20-Å average diameter is consistent with our model.

Dialyzed and undialyzed preparations yielded very similar solute diffusion rates, suggesting that the loss of heat modifiability (Fig. 3) did not result from the denaturation of the protein. Using maltose, lactose, sucrose, trehalose, and (GIY)₄ peptide in proteoliposome swelling assays, we failed to see any evidence of solute specificity for OmpG channels.

Outer membrane permeability of OmpG⁺ strains. We have shown above that OmpG produces a larger channel than OmpF or OmpC. Furthermore, OmpG is expressed in strain RAM194 at a level similar to that of the classical porins in other K-12 strains (18). We might expect that an abundant expression of a large-channel porin would make the cells hypersensitive to inhibitors and antibiotics. We therefore tested the susceptibility of strains DME553 (OmpC⁺), RAM123 (OmpC⁺ OmpG⁺), and RAM194 (OmpG⁺) to hydrophilic (ampicillin and chloramphenicol) and hydrophobic (novobiocin and rifampin) antibiotics. Cells expressing OmpG showed hypersensitivity to hydrophilic antibiotics: paper disks (7-mm diameter) presoaked with 10 μ g of ampicillin or 5 μ g of chloramphenicol gave inhibition zones of 13 mm in DME553 but around 18 mm in RAM123 or RAM194. In contrast, only a slight increase in sensitivity toward hydrophobic antibiotics was noted in cells expressing OmpG. These results further corroborated the above finding that the channel formed by OmpG is larger than those formed by the classical porins.

OmpG lacks stable oligomeric forms. The majority of porin proteins exist as homotrimers. However, the following experiments suggest that OmpG is monomeric porin. First, we carried out two-dimensional PAGE analysis (first dimension under native and second dimension under SDS-denaturing conditions) to look for evidence that OmpG is an oligomeric porin. We could detect OmpF trimers but no oligomeric forms of OmpG. Second, we carried out cross-linking experiments using dimethylsuberimidate and formaldehyde. In these experiments, either purified envelopes, OG-solubilized extracts, or whole cells were used. SDS-PAGE followed by immunoblot analysis did not reveal any evidence of cross-linked OmpG, although oligomeric forms were visible with OmpF (data not shown).

Expression of OmpG in other gram-negative bacteria. Immunoblot analysis using OmpG-specific antibodies showed the presence of a cross-reacting protein band with similar sizes in membranes of *S. flexneri* and *S. typhimurium* and even in *P. aeruginosa*, which does not belong to the family *Enterobacteri-*

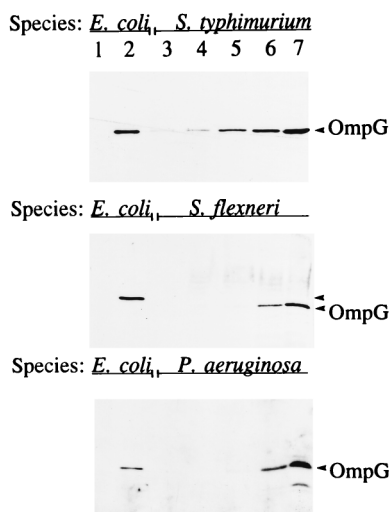


FIG. 5. Immunoblot analysis of OmpG from envelopes of various gram-negative bacteria. Lanes 1 and 2 contain envelopes (1.5 μ g of protein) from OmpG⁻ and OmpG⁺ *E. coli* strains, respectively; lanes 3 to 7 contain 0.75, 1.5, 3, 6, and 12 μ g of total protein, respectively. Gels were blotted with polyclonal antibodies raised against *E. coli* OmpG.

aceae (Fig. 5). In addition to being detected in *S. typhimurium* LT2, OmpG expression was detected in several clinical *Salmonella* isolates grown under our standard laboratory conditions (data not shown). It is curious that unlike in our *E. coli* strains, where OmpG is not expressed, it is produced, albeit apparently at a low level, in other bacterial species. It is conceivable that the lack of OmpG expression in our contemporary K-12 strains was due to chromosomal aberrations caused by the heavy mutagenesis used to construct these strains in the first place or that optimum growth conditions for its expression have not been found. Attempts to define growth conditions under which OmpG is expressed without mutations have so far been unsuccessful. To test the first hypothesis, we examined wild-type *E. coli* K-12 strains 679, Y10, W1, and AB1157, *E. coli* B, *E. coli* C, and *E. coli* K1 for OmpG expression by Western blot analysis. We failed to see OmpG from these strains. However, all strains yielded *ompG*-specific DNA fragments by PCR analysis.

Unlike *E. coli*, *S. typhimurium* contains three constitutively expressed porins, OmpD in addition to OmpF and OmpC (25). Immunoblot analysis using OmpG antibodies on envelopes prepared from OmpD⁺ and OmpD⁻ (*ompD*::Tn10) strains showed the presence of a cross-reacting protein band in both strains (data not shown). That OmpG is unrelated to OmpD was not surprising because we already knew that OmpG is unrelated to OmpF and OmpC, which show high antigenic and sequence conservation with OmpD (34, 35).

Regulation of OmpG: cloning of *cog*⁺. By using conventional genetic mapping methods, *cog-192* was mapped at 29 min on the chromosome (18). Diploid analysis revealed that *cog-192* was recessive, suggesting that it may disable the activity of a repressor protein that negatively controls *ompG* expression. However, the low frequency by which *cog-192* was isolated suggested that it is not a simple null mutation. The recessive nature of the *cog-192* mutation permitted the isolation of a plasmid clone containing the wild type *cog*⁺ gene from an *E. coli* gene bank by genetic complementation. The DNA insert carrying *cog*⁺ was roughly 10 kb long and thus likely to contain other genes that may or may not be relevant to *cog* function. The original *cog*⁺ clone was reduced to half its size by remov-

ing a restriction fragment. When introduced into an OmpG⁺ (Dex⁺) strain, this shorter clone still conferred an OmpG⁻ (Dex⁻) phenotype. Partial DNA sequencing from either end of the clone revealed the presence of truncated *aldH* on the one end and truncated *pspC* on the other. Four ORFs organized in the order *aldH*-*goaG*-*pspF*-*pspA*-*pspB*-*pspC* have been identified between these two genes (5, 11). Two smaller plasmid clones retaining both *pspA* and *pspB* (a 1.8-kb *Bgl*II-*Sal*I fragment) or just *pspA* (an 800-bp *Bgl*II-*Eco*RI fragment in which the *Eco*RI site was created immediately downstream from *pspA* by site-directed mutagenesis) also complemented the *cog-192* mutation. This showed a possible involvement of *pspA* in the regulation of OmpG expression.

Three additional observations strengthened the notion that *cog-192* allows OmpG expression by somehow affecting the *psp* operon: (i) both *cog-192* and *psp* map at 29 min on the *E. coli* chromosome, (ii) an introduction of Δ *pspA*::Km^r abolishes OmpG expression; and (iii) consistent with a proposal that *pspA* is autoregulated (41), we found that the presence of the *cog-192* mutation elevates the activity of a *pspA*::*lacZ* construct 10-fold.

Molecular characterization of *cog-192*. Complementation of *cog-192* by plasmid clones bearing just the *pspA* gene provided evidence that *cog-192* may define a chromosomal aberration affecting *pspA* gene function. When we used *pspA*-specific primers to amplify DNA from *cog*⁺ and *cog-192* strains by PCR, only the *cog*⁺ strain yielded the *pspA* fragment. The *psp* region was also examined by Southern blot analysis using three different probes, specific to *pspA*, *b1312* to *1313*, and *ompG* (Fig. 6). The data revealed that in the *cog-192* strain, regions corresponding to the *pspA* and *b1312* to *1313* probes were missing (Fig. 6). The *ompG*-specific probe revealed a substantial alteration in the genome immediately upstream to the *ompG* gene (Fig. 6).

***cog-192* defines a large chromosomal deletion.** In the *cog*⁺ strain, the *pspF* and *ompG* genes are separated by about 14.0 kb. Thus, under standard PCR conditions, it may not be possible to amplify a 14.0-kb intervening DNA fragment with primers specific to *pspF* and *ompG*. However, if this distance is significantly reduced, it should become possible to PCR amplify DNA by *pspF* forward and *ompG* reverse primers. To test this, we used two forward primers that were complementary to the *goaG*/*pspF* region and four reverse primers that were complementary to the *orf1*/*ompG* region (Fig. 6). As anticipated, no DNA fragments could be amplified from the *cog*⁺ strain. On the other hand, all but one set of primers yielded DNA fragments from the *cog-192* strain. Amplified DNA fragments, however, were much shorter than expected from a genome where the region between *pspF* and *ompG* was intact, showing that these two regions are now immediately adjacent to each other in the *cog-192* strain.

PCR-amplified DNA from the *cog-192* strain was used for nucleotide sequence analysis to pinpoint the deletion junction. The results showed that the region between *pspA* and *orf1*, including the last 369 (of 666) bp of the *pspA* gene and the first 685 (of 969) bp of *orf1* were deleted in the *cog-192* strain (Fig. 6). This showed that *cog-192* defined a large chromosomal deletion of a total of 13,127 bp between the *pspF* and *ompG* genes.

***cog-192* creates a transcriptional fusion between *pspA*' and *ompG*.** As mentioned above, the expression of OmpG in the *cog-192* strain was turned off by the presence of a *pspA*⁺ plasmid, indicating that PspA somehow controls OmpG expression in this strain. One explanation for this observation could be that the *cog-192* deletion resulted in *ompG* being placed under the control of a *pspA* promoter. Since the *pspA* promoter is

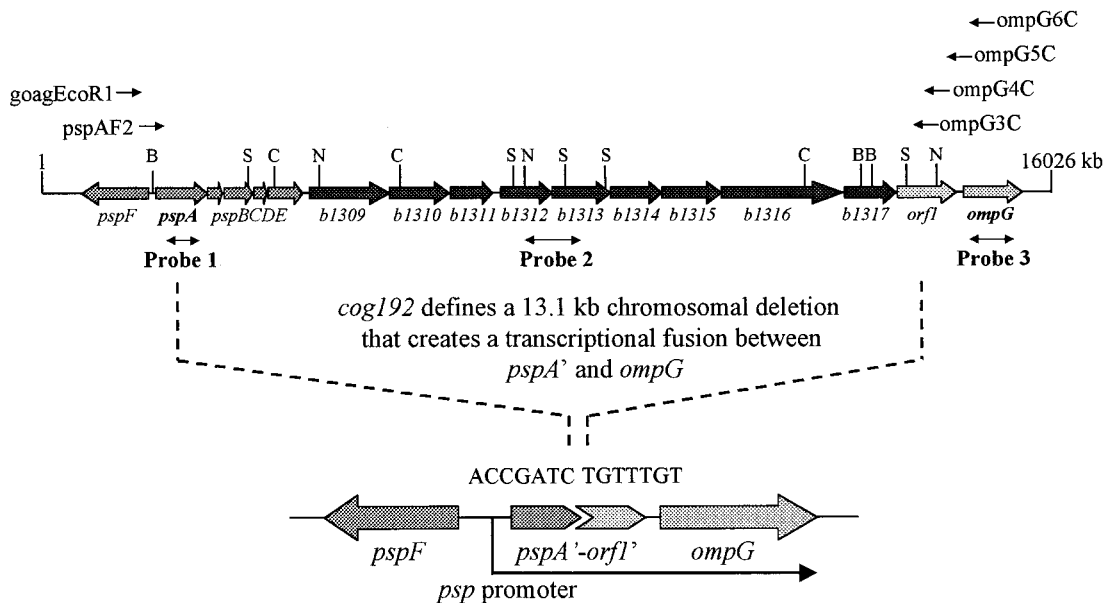


FIG. 6. Molecular organization of the chromosomal region encompassing *ompG* and the *psp* operon. Ten uncharacterized genes (*b1309* to *1317* and *orf1*) are present between *ompG* and the *psp* operon. These 10 ORFs as well as a significant portion of the *psp* operon are deleted (marked by dotted lines) in the *cog-192* strain. The deletion aligns *ompG* with the *pspA* promoter; precise ends of the deletion are shown in a close-up diagram below. Double-headed arrows show DNA probes used in Southern and/or Northern blot analysis. Locations of various primers and of restriction enzyme sites are shown at the top. Abbreviations: B, *Bgl*II; C, *Cla*I; N, *Nco*I; and S, *Sal*I.

regulated by the *pspA* gene product (41), the presence of a multicopy *pspA*⁺ plasmid would be expected to have a negative effect on its activity and hence *ompG* expression. This possibility was tested by Northern blot analysis of *ompG* mRNA (Fig. 7).

No *ompG*-specific transcripts were detected from a *cog*⁺ strain (Fig. 7; lanes 1 and 7). In contrast, two *ompG*-specific transcripts were detected from the *cog-192* strain (lanes 2 and 8). These transcripts were either absent (lane 5) or present in a greatly reduced amount (lane 6) when the *cog-192* strain was transformed with a plasmid bearing *pspFAB* or *pspA*, respectively. These results showed that a promoter that is negatively regulated by the *pspA* gene product controls *ompG* transcription in the *cog-192* strain. Northern analysis also revealed the presence of two *ompG*-related transcripts that were regulated in the same fashion (Fig. 7). One interpretation of this observation is that the two transcripts initiated from the same promoter but were terminated at different points.

Chromosomal deletions are necessary for OmpG expression. We sought additional Dex⁺ revertants from DME553 (*cog*⁺ Dex⁻) to examine whether mutations other than deletions could permit OmpG expression. Dex⁺ revertants were obtained from 10 independent cultures. Dex⁺ isolates expressing OmpG were estimated to be around 1% of the total Dex⁺

revertants obtained, as judged by Western blotting. PCR and Southern analyses of the chromosomal DNA from five independently obtained Dex⁺ isolates revealed deletions upstream of *ompG*. In two isolates, the deletion extended beyond the entire *psp* operon; in three, the deletions retained *pspA*. As was the case for the *cog-192* strain, OmpG expression in the latter three isolates was inhibited by a plasmid carrying *pspA*⁺. These results support the notion that deletions upstream of the *ompG* structural gene are necessary for OmpG expression under laboratory growth conditions. These deletions must create novel junctions between *ompG* and an upstream promoter element that may not be genetically related to *ompG* in the parental strain.

DISCUSSION

The molecular and biochemical analyses presented in this study showed that OmpG is a unique class of porin. Consistent with our previous *in vivo* findings (18), the *in vitro* data revealed that OmpG channels are functionally larger than those of the classical porins. The major reason for large OmpG channels may be the absence of a large external loop, L3, which in classical porins folds back into the channel and serves to restrict pore size (6). Since the distance between neighboring strands in a β sheet is 0.45 nm, the 16-membered β barrel predicted for OmpG (Fig. 2) will produce a channel with the diameter $0.45 \times 16/\pi = 2.3$ nm. Considering that side chains will narrow the channel somewhat, this value is in perfect agreement with the diameter determined by the solute size dependence of sugar flux (Fig. 6). If our model is correct, OmpG is the first porin with such a simple barrel structure.

Large OmpG channels mean that a greater variety of nutrients can be taken up by the bacteria, which would provide a growth advantage to bacterial strains producing OmpG. Yet, OmpG expression has not been observed in wild-type *E. coli* strains obtained from laboratory collections. In contrast,

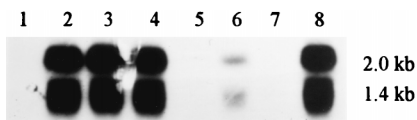


FIG. 7. Northern blot analysis to detect *ompG*-specific transcripts. Total RNA (3 μ g) obtained from DME553 (*cog*⁺; lanes 1 and 7), RAM123 (*cog-192*; lanes 2 and 8), RAM123 *recA*::Km^r (lane 3), RAM123 *recA*::Km^r/pBR322 (lane 4), RAM123 *recA*::Km^r/pBR322-*pspFAB* (lane 5), and RAM123 *recA*::Km^r/pBR322-*pspA* (lane 6) was probed with an *ompG*-specific probe. Molecular sizes of the two *ompG*-specific mRNA species are 1.4 and 2.0 kb.

OmpG expression has been observed in *Salmonella* and *Shigella* strains analyzed so far. The level of expression, however, appears to be in the range of 10 to 20% of the levels of classical porins, which may explain why the presence of this porin was not noticed earlier. Future work should address the significance of OmpG in these organisms. It is conceivable that OmpG was a functional porin in the ancestor of present day *Enterobacteriaceae* and that its expression was down regulated or abolished in *E. coli*, which confronts the constant challenge of toxic bile salts (39).

The discovery of OmpG showed the potential of bacterial genomes to code for a number of porin proteins. Of course, not all porins are expressed at the same time. Some, such as general porins, may be required under all growth conditions, although their relative levels fluctuate in response to physiological parameters. These proteins are present in large quantities (around 10^5 molecules/cell). However, solute diffusion through OmpF and OmpC channels is not efficient, which may have contributed to the evolution of bacteria with specialized porins, such as LamB and Tsx, which are needed only under conditions of specific nutrient availability. Solute specificity for OmpG channels and physiological parameters regulating its expression are not known.

OmpG expression was achieved under laboratory conditions where the entry of large sugars was not possible through general channels. A mutation, *cog-192*, permitting OmpG expression turned out to be a chromosomal deletion that removed 13.1 kb of DNA immediately upstream of *ompG*. This deletion placed *ompG* expression under the control of the *pspA* promoter. The regulation data contrasted with our previous hypothesis that operator or repressor mutations derepress OmpG expression (18). Indeed, the recessive nature of *cog-192* and plasmid complementation data supported a repressor theory. Molecular analysis of *cog* mutations, however, demonstrated that regulated expression of OmpG was the result of a novel gene fusion. Jacob and colleagues (1, 9) reported deletions fusing two unrelated operons where the downstream genes were also placed under the transcriptional control of an upstream promoter for unrelated operon. Computer analysis of the nucleotide sequence upstream of *ompG* did not reveal the existence of a σ^{70} -dependent promoter element. It is conceivable that *ompG* and 10 other ORFs sandwiched between it and the *psp* operon are not expressed in *E. coli* due to the absence of an active promoter.

Sequence homology data showed that several of these 10 uncharacterized ORFs may code for proteins involved in the transport or metabolism of sugars (5). Interestingly, *orf1*, located immediately upstream of *ompG*, shows a strikingly high identity with the ATPase component of various ABC transporters (7). These components are usually associated with other proteins dedicated to the transport of a specific substrate, and genes coding for these other components are present in the vicinity of *orf1*. At this point it is unclear whether *orf1* and *ompG* are part of the same operon. Our data showed that the OmpG-mediated Dex⁺ phenotype is dependent on the *malK* gene product of the *mal* operon but not on *orf1*. Curiously, we found that *orf1* sequences from *E. coli* B and *E. coli* C strains had the capacity to encode a 360-residue protein (compared to the 322-residue Orf1 from K-12). A truncation of Orf1 in *E. coli* K-12 was due to the presence of a natural frameshift mutation (deletion of G) close to the end of the gene. The truncation of Orf1 may render the protein nonfunctional and/or highly susceptible to proteolysis.

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