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Overexpression of Outer Membrane Porins in *E. coli* Using pBluescript-Derived Vectors

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The genes coding for four major outer membrane porins of *Escherichia coli, ompF, ompC, phoE,* and *lamB,* have been cloned into pBluescript-derived vectors and overexpressed to very high level (approximately 80% of the total membrane protein) in widely used host strains lacking one or more porins. For OmpF, OmpC, and PhoE porins it is shown that, contrary to current dogma, the genes can be overexpressed without undue deleterious effects upon cell growth and are stable, even under conditions of continuous expression. In contrast, overexpression of LamB is toxic to cell growth, but can be performed using tightly regulated *lac* promotor-driven expression. The vectors described allow overexpression, sequencing, and mutagenesis to be performed using a single system, without the necessity of subcloning, thus simplifying genetic manipulation. A particular advantage of these new vectors (with the exception of the vector for LamB) is that they do not require a particular regime for inducing the recombinant protein. To our knowledge, this study is the only comparative study of widely used membrane porin expression systems and the first to show that several porins can be stably expressed individually and maintained on high copy number vectors.

Outer membranes Gene expression Overexpression Porins

HIGH-LEVEL expression of outer membrane porins is becoming increasingly interesting due to their application in the production of vaccines by epitope insertion into extracellular loops [for reviews see (7,14,37,47)]. The elucidation of the three-dimensional structures at the atomic level of three major porins of *E. coli* (11,41) as well as those of two outer membrane porins from *Rhodobacter capsulatus* (51,52) and *Rhodopseudomonas blastica* (26) opens the possiblility of rational site-directed mutagenesis to study structure–function relations. In addition, structural elucidation of other porins from pathogenic gram-negative bacteria is an aid to rational vaccine design.

Membrane protein structural elucidation by X-ray crystallography of three-dimensional crystals or by electron crystallography of two-dimensional crystals is clearly aided by overexpression, with respect to the ease of purification and as well as enhancing crystal quality. For the major outer membrane porins of *E. coli* (OmpF, PhoE, OmpC, and LamB) several different vector host systems have been employed for overexpression, all of which are based upon intermediate copy number vectors (pBR322, pACYC184) and with gene expression driven by either a strong, constitutive endogenous promotor (e.g., OmpF, PhoE, OmpC) or an inducible, medium strength promotor (e.g., the *tac* promotor-driven expression of the *lamB* gene). These expression systems have been devised by different groups with goals other than crystallization and direct structural elucidation, and to our knowledge, no comparison of the the efficiency of these systems has been performed. In addition, the use of intermediate copy number vectors necessitates tedious subcloning steps for site-directed mutagenesis and sequencing. This strategy has been dictated by the notion that porin genes are unstable and toxic when cloned into high copy number vectors.

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In this study, we show that at least four major porins from *E. coli* can be stably cloned into pUC-derived vectors and expressed at very high level, possibly at the highest attainable expression limits of the outer membrane. In all cases, a single vector can be used for site-directed mutagenesis and expression, thus reducing the time required for these steps significantly. Finally, this study is the first to compare different expression systems of the above-mentioned porins under identical conditions.

MATERIALS AND METHODS

Growth of Strains for Cloning and Expression

In general, all strains of *E. coli* were grown using LB medium with antibiotic selection with concentrations: tetracycline, 10 μg/ml; kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml. For expression studies, cells were freshly transformed with purified plasmid immediately prior to the experiment.

Biochemical Techniques

The isolation of outer membranes was performed as follows. Cells grown in 100-ml cultures to the late exponential phase were harvested by centrifugation at 7000 rpm in GSA flasks for 30 min at 4°C. Pellets were washed with 50 mM Tris-HCl, pH 8.0, and then resuspended in 20 ml of the same buffer containing 100 μM phenylmethanesulfonylfluoride (PMSF) and 2 μg DNAase. After cell breakage by a single passage through a French Press (Aminco), unbroken cells were removed by centrifugation at 7000 rpm for 20 min at 4°C, then the crude outer membranes sedimented by further centrifugation at 18,000 rpm for 20 min. Outer membranes were resuspended with the above buffer and homogenized with a precision Potter homogenizer and aliquots taken for protein determinations (Bio-Rad). Samples (20 μg) were analyzed using SDS-PAGE according to Laemmli (27) and stained with Coomassie Blue. In some cases, the relative intensities of various bands were determined using a densitometer (Molecular Dynamics, model 300A).

Alkaline extraction was performed with purified outer membranes using 100 mM $NaHCO_3$, pH 11.5, as described (13). Trypsin treatment of whole membranes and purified LamB was performed by adding *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Worthington, USA) dissolved in 1 mM HCl to 20 μg protein in 0.5 ml 50 mM Tris-HCl, pH 8.0, containing 5 mM $CaCl_2$ to give a final concentration of 100 μg protein/ml (7,40). After further incubation at 37°C for 1 h, 50 μl 72% trichloroacetic acid (TCA) was added and the samples vortexed. After incubation for 45 min at room temperature, samples were pelleted by centrifugation at $3000 \times g$ for 20 min, washed once with 0.5 ml 20 mM Tris-HCl, pH 8.0, and the pellet resuspended in SDS sample buffer for SDS-PAGE analysis. Purified LamB was obtained as described (46). Western blotting and detection using a horseradish peroxidase-coupled secondary antibody was performed according to Towbin et al. (50) using a polyclonal antiserum raised against trimeric LamB (Y. F. Wang and J. P. Rosenbusch, unpublished data).

Construction of pBluescript-Derived Porin Expression Plasmids

DNA manipulations were performed as described (39). Single- and double-stranded sequencing was performed using the Sequanase Version 2.0 (USB) according to the manufacturer's protocol. In all cases below, the insert of the DNA in the final expression vector was partially sequenced to confirm its identity.

pBsOMPF1. The pBR322-derived parental plasmid, pMY222 (36), was cleaved with *Ava*I and religated, then transformed into RR28 (18) to yield the plasmid pYG9. pYG9 is identical to plasmid pGR203 (36), but lacks the *Xba*I linker. pYG9 was then restricted with *Dde*I, and treated with the *Pol*Ik. The 1.8-kb fragment (Fig. 1), encoding the *ompF* gene (22), was isolated after separation by gel electrophoresis. The 1.8-kb fragment was then ligated to pBluescript KS+ (pBs), previously cleaved with *Sma*I and dephosphorylated, to yield the plasmid pBsOMPF1 after transformation into *E. coli* RR28 and reisolation. In this construction the *ompF* promotor and coding sequence are in the opposite orientation to the *lacUV5* promotor of pBs.

pBsPHO1 and pBsPHO2. A 1.5-kb *Eco*RV fragment (Fig. 1) containing the *pho* promotor region and *phoE* coding sequence (32) was cleaved from the pACYC184-derived parental plasmid, pJP29 (4) and isolated by gel electrophoresis. The fragment was then ligated with pBs cleaved with *Eco*RV and dephosphorylated and transformed into XL1-Blue. After transformation the plasmids pBsPHO1 and pBsPHO2 were obtained, containing the *phoE* gene either in the opposite or the same orientation to the *lacUV5* promotor of pBs, respectively.

pBsOMPC1. The pBR322-derived parental plasmid, pMY150 (29) was cleaved with *Xba*I/*Hin*dIII, treated with *Pol*Ik in the presence of dNTPs to gener-

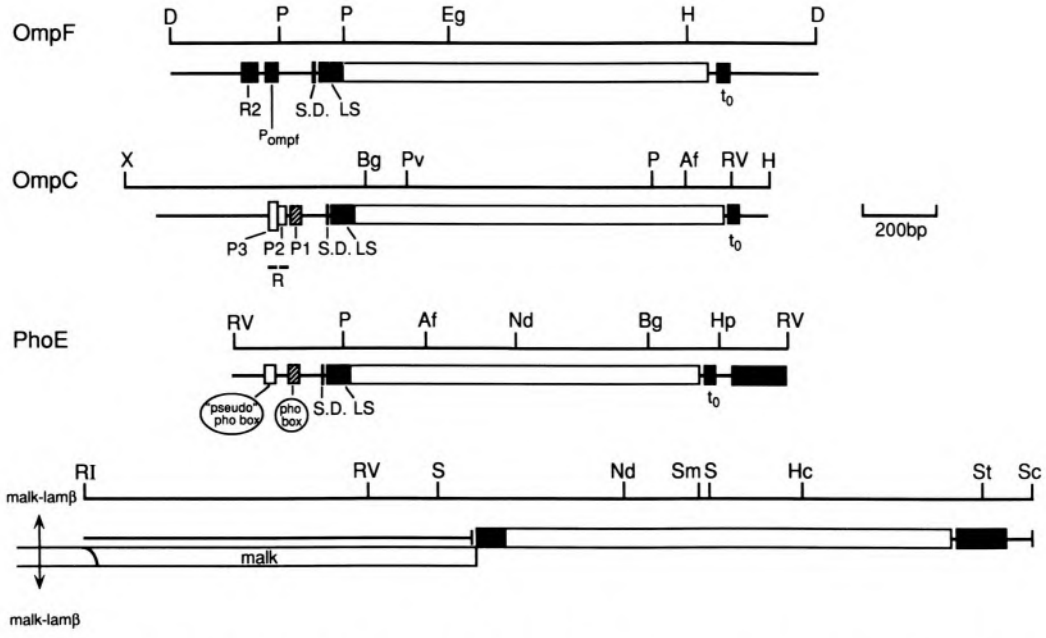

FIG. 1. The cloned porin-encoding fragments together with their promotors (P), inserted into pBs-derived expression vectors, Shine-Delgarno ribosomal binding sites (rbs), and terminators (t_0) are shown. *ompF*: The constitutive promotor (p_{ompf}) as well as the OmpR2 binding site are depicted as closed rectangles (22,30,31,44,45). The weak *cis*-regulatory site −450 bp upstream from the transcriptional start (20) is not present on the *Dde*I fragment; *ompC*: three potential promotor regions (P1, P2, P3) (21,31,28) are shown. The OmpR binding sites are shown as open rectangles underneath the promotors. *phoE*: the positions of the "*pho* box" and "pseudo-*pho* box" consensus sequences of the phosphate-inducible promotor are shown (49). *malk-lamB* (2): the 2.65-kb *Eco*RI-*Sac*I fragment obtained from pHCP2 encoding part of the *malk* gene (malk) and the full-length *lamB* gene is shown. This fragment contains no endogenous promotor. The *malk-lamB* fragment is under control of the *lacUV5* promotor provided by the *lacZ*$^{\alpha}$ gene of pBs. Several potential stop codons in the *malk* gene prevent the expression of a possible *lacZ*$^{\alpha}$-*malk* fusion product. Restriction enzymes: D, *Dde*I; P, *Pst*I; Eg, *Eag*I; H, *Hin*dIII; Xb, *Xba*I; Bg, *Bgl*II; Pv, *Pvu*II; Af, *Afl*III, RV, *Eco*RV; Nd, *Nde*I; Hp, *Hpa*I; RI, *Eco*RI; S, *Sal*I; Nc, *Nco*I; St, *Stu*I; Sc, *Sac*I; Sm, *Sma*I.

ate blunt ends, and the *ompC* coding sequence and the *ompC* promotor region isolated as a 1.7-kb fragment (Fig. 1) by gel electrophoresis. pBs was cleaved with *Sma*I and dephosphorylated, then ligated with the 1.7-kb fragment from pMY150, to generate the plasmid pBsOMPC1 after transformation. The *lac* promotor of pBsOMPC1 is in the reverse orientation to the coding sequence of *ompC*.

pBsLAMB1. The pBR322-derived plasmid pHCP2 (9) was cleaved with *Eco*RI and *Sac*I to yield a 2.6-kb fragment containing the *lamB* gene (8) together with its terminator and an upstream 0.95-kb region corresponding to part of the gene *malk* (Fig. 1). The *Eco*RI-*Sac*I fragment was ligated with pBs, which had been previously cleaved with the same enzymes, to yield the plasmid pBsLAMB1, with the *lamB* gene thus under control of the *lac* promotor of pBs. The partial *malk* region contains several potential stop codons in-frame with the lacZ$^{\alpha}$ fragment. For some experiments, pBsLAMB1 was placed under *lacI*^{q} control by using the compatible plasmid pACYCLACIQ1. The latter was constructed by blunt-end ligation of a *Pol*Ik-treated 1.1-kb *Eco*RI fragment obtained from pAC1 (5), containing the *lacI*^{q} gene, into the *Pol*Ik-treated *Hin*dIII/*Sal*I sites of the *tet* gene from pACYC184 (6).

Expression strains lacking one or more porins were obtained from their original sources and grown with the appropriate selection (Table 1).

RESULTS

Plasmid Stability and Choice of Suitable Expression Strains

All of the plasmids, pBsOMPF1, pBsOMPC1, pBsPHO1, and pBsPHO2, appeared to be stable in both cloning and expression strains. Expression experiments were only performed using freshly transformed cells. It was noticed, however, that only certain strains were suitable for cloning. All of the above plasmids were stable in the strains XL1-Blue, allowing high cell densities and good yields of both double- and single-stranded DNA [after superinfection with the helper phage VCSM13 (Stratagene)] to be achieved. In the cases of pBsOMPC1 and pBsOMPF1, however, cloning steps were usually performed using

TABLE 1
PLASMIDS AND STRAINS USED IN THIS STUDY

	Determinants	Reference
Plasmids		
pBs	pBluescript KSII+, Ap^R, *lacZ*$^{\alpha}$, f1(+) intergenic region	Stratagene
pACYC184	Cm^R, Tet^R, p15-A replicon	Chang and Cohen (6)
pBR322	Ap^R, Tet^R, colE1 replicon	Bolivar (3)
pMY222	pBR322 derivative containing a 6.2-kb *Eco*RI-*Hin*dIII fragment encoding *ompF* and flanking regions, Ap^R	Ramakrishnan et al. (36)
pYG9	pMY222 digested with *Ava*I and religated to remove the 4.7-kb *Ava*I fragment downstream of the *ompF* gene, Ap^R	This study
pBsOMPF1	pBs derivative containing a 1.8-kb *Dde*I fragment encoding*ompF* from pYG9, inserted into the *Sma*I site of pBs, Ap^R	This study
pMY150	pBR322 derivative containing a 1.7-kb *Hin*dIII-*Sal*I fragment encoding the *ompC* gene, Ap^R	Mizuno et al. (29)
pBsOMPC1	1.7 kb *Xba*I-*Hin*dIII fragment from pMY150, encoding *ompC*, inserted into the *Sma*I site of pBs, Ap^R	This study
pJP29	pACYC184 derivative, containing a 1.5-kb insert encoding *phoE*, Cm^R	Overbeeke et al. (32)
pBsPHO1	1.5-kb *Eco*RV fragment obtained from pJP29, encoding *phoE*, into the *Sma*I site of pBs in the reverse orientation as the *lac* promotor, Ap^R	This study
pBsPHO2	1.5-kb *Eco*RV *phoE*-encoding fragment inserted in same orientation as the *lac* promotor of pBs, Ap^R	This study
pHCP2	pBR322-derived vector containing a 2.6-kb fragment encoding *lamB* and the last 0.95 kb of coding sequence of *malK*, Ap^R	Clement et al. (9)
pBsLAMB1	*Eco*RI-*Sac*I 2.6-kb fragment encoding part of *malK* and *lamB*, derived from pHCP2, inserted into the corresponding sites of pBs, Ap^R	This study
pAC1	pBR322-derived, *lacI*^{q}, *lamB* gene under control of the *tac* promotor, Ap^R	Boulain et al. (5)
pACYCLACIQ1	pACYC184 derivative, containing a 1.1-kb fragment encoding *lacI*^{q}, inserted into the *Hin*dIII-*Sal*I sites of the *tet* gene	This study
Strains		
XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^R)]	Stratagene
RR28	*supE44, hsdR hsdM recA pheS12 thi leu pro lac gal ara ntl xyl endA*	Hennecke et al. (18)
DH5α	*supE44* ΔlacU169, (F80*lacZΔM15*) *hsdR17 recA1 gyrA96 thi-1 relA1*	
CE1248	*thi ΔphoE-proAB pyrF thyA argG ilvA mal tonA phx rpsL deoC sup recA56 vtr glpR ompR phoR69 phoA8*	Korteland et al. (25)
BZB1107	*E. coli* B^s, *ompF*::Tn*5*, Km^R, Str^R	A. P. Pugsley, unpublished
pop6510	*thr, leu, tonB, thi, lacY1, recA*::Tn*10*, dex5, metA, supE	

Ap^R, resistance to ampicillin; Cm^R, resistance to chloramphenicol; Km^R, resistance to kanamycin; Str^R, resistance to streptomycin; Tet^R, resistance to tetracycline.

the strain RR28 (18), which remains very viable after transformation with recombinant porins and yields very high cell densities and good quality double-stranded DNA. Preliminary results indicate that the *sec* machinery in *E. coli* RR28 is defective, which may prevent the expression of porins at high level in the outer membrane. The low level of porin expression may thus reduce the potentially toxic effects of these proteins, thus indirectly enhancing plasmid stability. By contrast, strain DH5α appeared to be highly sensitive to the presence of all recombinant porin vectors employed, leading to very slow growth and low cell yields.

All cloning strains expressing LamB from pBsLAMB1 showed toxic side effects. Such effects were obviated by coexpressing the *lacI*^{q} gene by cloning into RR28 or XL-1 containing the compatible plasmid, pACYCLACIQ1 (see below).

The choice of appropriate expression strains was critical. Thus, the expression of LamB using either the strong *tac* (pAC1) or *lac* (pBsLAMB1) promotors, respectively, was performed using the strain pop6510. In both cases, high levels of $LacI^q$ were beneficial for the maintenance of plasmid stability and achieving high expression levels of the desired porin. The strain pop6510 (dex^-) is highly tolerant to high-level expression of *lamB*, though cell densities achieved after induction are about half that observed without induction, the cells showing little tendency to lyse or desiccate upon streaking. This does not apply for CE1248 nor BZB1107 (usually employed for PhoE or OmpF expression, respectively), both of which show growth parameters typical of toxic genes (data not shown). We also observed that the levels of PhoE expressed were highest for CE1248, which is *ompR*$^-$ [hence phenotypically *ompF*$^-$/*ompC*$^-$ (15–17)]

and constitutively expresses *phoE* from the *pho* promotor due to the deletion of the "*pho* box" regulatory genes (*phoS, phoT, phoR*) (25). OmpF expressed from pMY222 or pBsOMPF1 appears toxic for CE1248 but is expressed at comparable levels in both BZB1107 and pop6510, or in the methionine auxotroph DL41, which is often used for the production of selenomethionine-substituted proteins (53).

Estimation of the Relative Expression Levels of Recombinant Porins

We have estimated the relative expression of the different porins by visual and densitometric comparison of the observed total monomers produced after boiling with the amount of OmpA present in the membrane. It has been shown by several groups (10,12,29,35) that, due to the limiting space present in the outer membrane, the level of OmpA is inversely related to the overexpression of a given recombinant porin.

Expression of OmpF and OmpC in the Expression Strain BZB1107

Figure 2a shows the growth curves for the plasmids pMY222, pYG9, and pBsOMPF1, coding for OmpF, and the plasmids pMY150 and pBsOMPC1, coding for OmpC, respectively, using the expression strain BZB1107. The parental strain without plasmid was used as a control. BZB1107 is a derivative of *E. coli* B^E containing a Tn*5* insertion at the beginning of the *ompF* gene (A. Prilipov and J. P. Rosenbusch, unpublished data). BZB1107 does not express the porin OmpC (38).

Expression of the *ompF* gene from the plasmid pMY222 enhanced the growth rate of BZB1107 relative to the growth in the absence of plasmid. Comparison with the SDS-PAGE profile of the outer membranes (Fig. 3a) shows that the *ompF* trimer was overexpressed by a factor of two- to fourfold if compared to a strain with a single chromosomal gene (data not shown). By comparison, BZB1107(pYG9) grew similarly to the parental strain and and revealed a reduction in the expression level of *ompF* when compared to pMY222. We cannot explain this observation at present as all known regulatory elements present on pMY222 are present on pYG9. Possibly the deleted downstream sequences on the latter plasmid may play a role in regulating the high level of expression observed for pMY222. The pBs derivative, pBsOMPF1, increased the generation time of BZB1107 by a factor of 1.6 in comparison to BZB1107 without plasmid and BZB1107 (pMY222) (see Table 2), and the final cell density achieved was usually about 20% lower than for BZB1107-(pMY222). Analysis of the SDS-PAGE profile from outer membranes obtained in the late exponential phase showed that the amount of OmpF produced was increased 1.5- to 2-fold compared to BZB1107-(pMY222) and that the amount of OmpA was correspondingly reduced.

The effect of overexpression of the *ompC* gene from a pBs-derived plasmid was even more striking (Fig. 2b). Whereas the pBR322-derived plasmid pMY150 grew identically to BZB1107 without plasmid, BZB1107(pBsOMPC1) showed a long lag period, although the generation time in the exponential phase was almost the same (Table 2) as that of BZB1107(pMY150). However, analysis of the SDS-PAGE profile showed that BZB1107(pBsOMPC1) expressed approximately twice the level of OmpC observed for BZB1107(pMY150). In both cases, the amount of OmpA was reduced in comparison to the same strain in the absence of plasmid.

Expression of PhoE in the Expression Strain E. coli *K12 CE1248*

Figure 2c depicts the growth curves obtained for the plasmids pJP29 (4), pBsPHO1, and pBsPHO2 in the expression strain CE1248. Growth rates are compared together with that for CE1248 in the absence of plasmid. The strain CE1248 is *ompR*$^-$ and thus lacking detectable levels of OmpC and OmpF. Because CE1248 is also *phoS*$^-$/*phoR*$^-$, it constitutively expresses the *phoE* gene under the control of the *pho* ["*pho* box" (49)] promotor. The slow growth rate of the parental strain, lacking the major porins, was significantly increased when PhoE was expressed from either pJP29 or the pBs-derived plasmids pBsPHO1 and pBsPHO2 (Table 2). Interestingly, the SDS-PAGE profile (Fig. 3b) showed that pBsPHO2, containing the *phoE* gene oriented in the opposite direction to the lac promotor of pBs, yielded approximately twice the amount of PhoE porin in the outer membrane to that of pJP29. In addition, despite the 5- to 10-fold increased copy number of pBsPHO1 compared to that of pJP29, only a two- to fourfold increase of PhoE expression was observed (Table 2).

Expression of Maltoporin in the E. coli *K12 Expression Strain pop6510*

Figure 2d shows the growth curves obtained with the pBR322-derived plasmid pAC1 or the pBs-derived plasmid pBsLAMB1 when expressed in the *E. coli* K12 derivative pop6510 (*dex*$^-$). As pAC1 is under strong repression from *lacI*^{q} present on the same plasmid (5), the cultures were induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) when the A_{660} (4-mm path length) reached 0.25. No IPTG

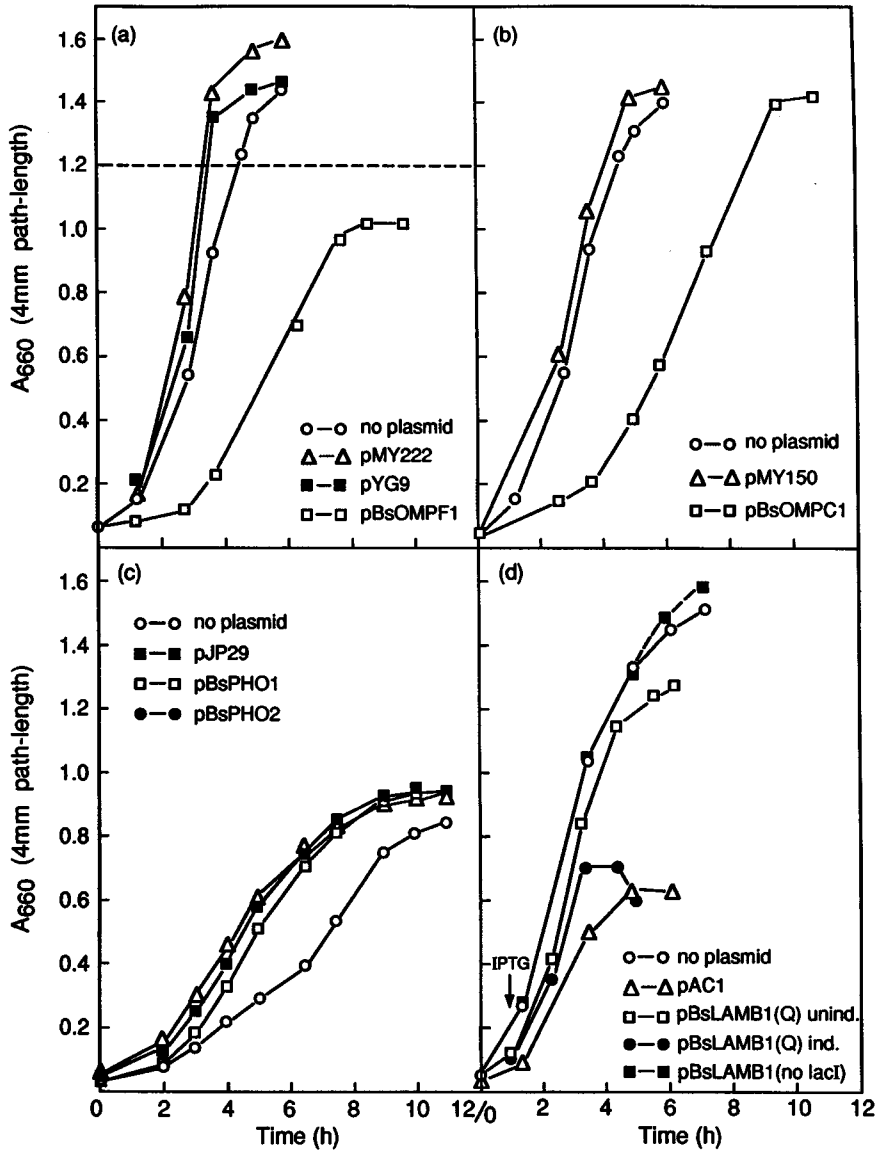

FIG. 2. Growth curves for the expression strains transformed with the porin expression plasmids (indicated on the figure) are shown: (a) and (b) strain BZB1107; (c) strain CE1248; (d) strain pop6510. pBsLAMB1 and pBsLAMB1(Q) refer to expression experiments performed in the absence or presence of the compatible plasmid pACYCLACIQ1.

was added to the culture of pop6510(pBsLAMB1), because the single chromosomally encoded *lacI* gene of this strain is insufficient to repress the *lac* promotor. The SDS-PAGE profiles shown in Fig. 3d confirm that pAC1 is very efficient in expressing *lamB*, although the SDS-PAGE profile of the unboiled sample shows a large number of incorrectly assembled monomers. Similar profiles have been observed previously (47). For pAC1 the high expression level observed is clearly toxic to the cells, as the growth rate was abruptly decreased after induction with IPTG, in comparison to the controls, and the final cell densities obtained were much lower than those observed for pop6510 in the absence of plasmid (Table 2).

By comparison, the expression of *lamB* from pBsLAMB1 is not clearly visible by SDS-PAGE, although biochemical studies in our laboratory have shown that trimeric LamB can be purified from this strain by starch affinity chromatography (24). In contrast to the other pBs plasmids mentioned above, plasmid analysis of the late exponential phase revealed a number of deletion derivatives of pBsLAMB1 (in contrast to pAC1, which remains stable). In addition, colonies expressing pBsLAMB1 flattened and dried during prolonged incubation at either 37°C or 4°C. This was not observed for the pBs-derived plasmids described above. Compatible with the low level of expression of pBsLAMB1 was the observation that the

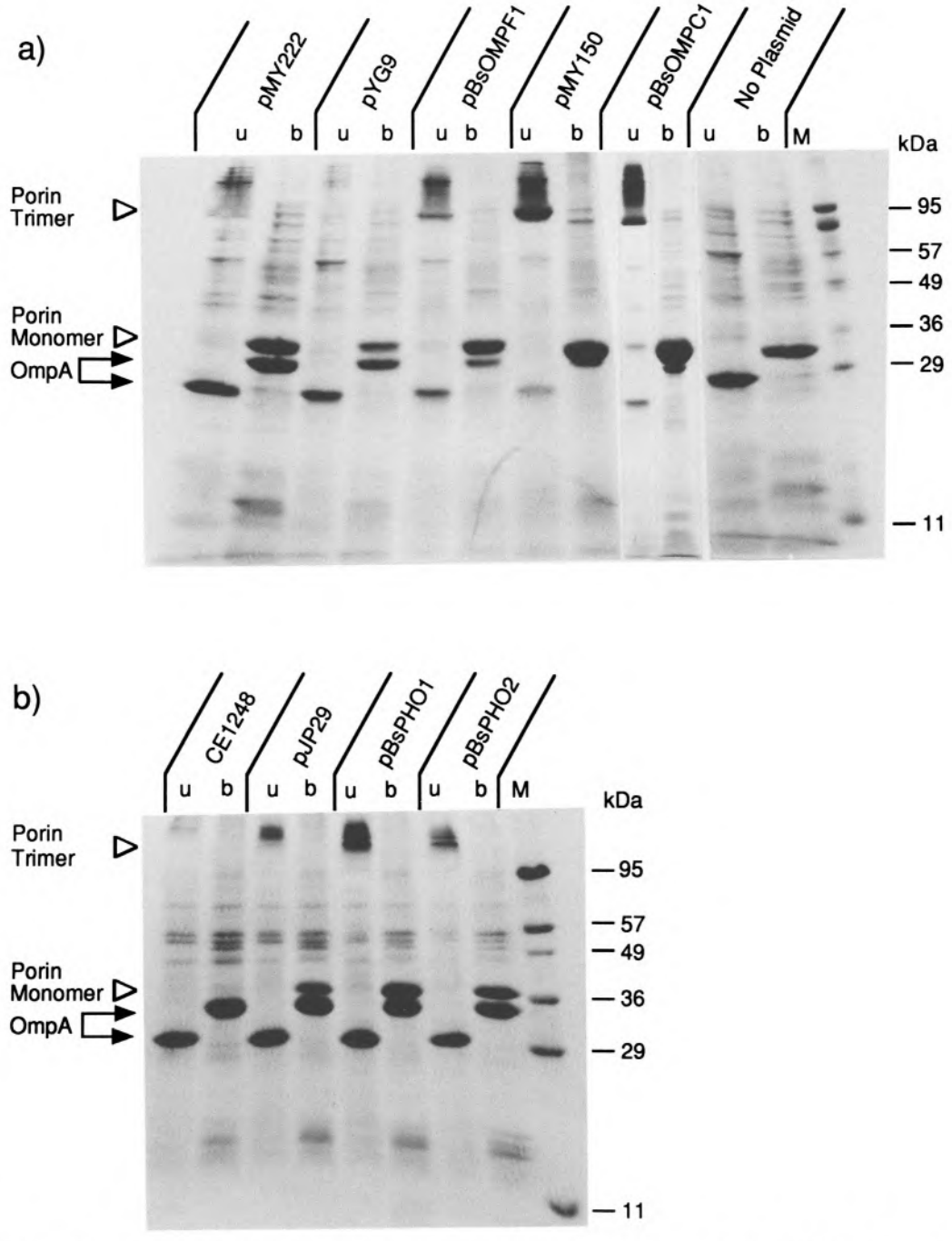

FIG. 3. SDS-PAGE profiles of purified outer membranes harvested from late exponential cultures of either (a) BZB1107 containing either *ompF*- or *ompC*-encoding expression plasmids, or (b) CE1248 containing *phoE*-encoding expression plasmids. In all cases, a control without plasmid is shown for comparison and all preparations were either boiled (small b) or unboiled (small u) before application to the gel. The expressed porin (p) is indicated and the position of OmpA before (lower arrow) or after (upper arrow) is indicated. In general, the amount of protein applied to the gel was adjusted to contain similar amounts of OmpA, thus facilitating a visual comparison of the porin expression levels. However, the relative amounts of expressed porin were quantitated by densitometric analysis. Thus, although the amount of OmpF expressed from pBsOMF1 appears visually somewhat less than that of pMY222 (the parental plasmid), this is due only to the smaller amount of protein applied and was not confirmed by densitometry (Table 2).

TABLE 2
SUMMARY OF RESULTS OBTAINED USING DIFFERENT PORIN EXPRESSION VECTORS

Expression Strain	Plasmid	Promotor*	Plasmid Derivative†	Expression Mode	Generation Time ($t_{1/2}$, min)	Cell Density Achieved by pBs/Parental Plasmid‡	Expression Level Compared to OmpA§
BZB1107	none	none	none	none	54	none	none
	pMY222	p_{ompF}	pBR322	constitutive	49	1.0	1.0
	pYG9	p_{ompF}	pBs	constitutive	54	1.0	0.5
	pBsOMPF1	p_{ompF}	pBs	constitutive	82	0.8	1.52
	pMY150	p_{ompC}	pBR322	100 min	110	1.0	~2.0
	pBsOMPC1	p_{ompC}	pBs	100 min	104	1.0	~2.1
CE1248	none	none	none	none	155	1.0	1.0
	pJP29	p_{pho}	pACYC184	inducible	84	1.0	2.5–4.0
	pBsPHO1	p_{pho}	pBs	inducible	73	1.0	2.5–4.0
	pBsPHO2	p_{pho}	pBs	inducible	103	1.0	2.5–4.0
pop6510	none	none	none	n.d.	n.d.	n.d.	n.d.
	pAC1	tac	pBR322	inducible	n.d.	1.0	1.0
	pBsLAMB1	p_{lac}	pBs	inducible/constit.	n.d.	3.0	0.2
	pBsLAMB1Q	p_{lac}	pBs	inducible	n.d.	1.2	1.0

*See Fig. 1.

†Little or no change of the reported plasmid copy number (pBR322, pACYC184, 20–50 copies/cell; pBs, 200–500 copies/cell) was observed in this study.

‡+(−) no significant change in attainable cell density comparable to the strain containing the expression plasmid.

§Estimated by densitometry of Coomassie Blue-stained SDS-PAGE profiles after normalizing to the level of OmpA.

growth profile for pop6510(pBsLAMB1) was identical to that of pop6510 alone. Attempts to select for the *lamB* gene using 0.4% maltotriose as the single carbon source were unsuccessful, low-level expression of the gene being sufficient to allow the transport of maltotriose into the cell.

As spontaneous deletions in a recombinant gene often indicate toxicity, we attempted to bring the expression of pBsLAMB1 under tight control by performing expression experiments in the presence of the *lacI*^{q} gene, present on the pACYC184-derived plasmid, pACYCLACIQ1. After induction by the addition of IPTG, the growth of pop6510(pBsLAMB1/pACYCLACIQ1) was markedly slowed and SDS-PAGE profiles of outer membrane preparations indeed revealed the expression of both trimeric and monomeric LamB at levels identical to those observed for pop6510(pAC1). The presence of the *lamB* gene products was confirmed by immunoblotting (Fig. 4). Finally, we tested whether the monomeric LamB was loosely bound to the outer membranes obtained from pop6510(pAC1) and pop6510(pBsLAMB1/pACYCLACIQ1) by extraction with 2% SDS at room temperature. The SDS-PAGE profiles from membranes before or after extraction showed no reduction of the intensity of the monomer (data not shown) and treatment with alkaline Na_2CO_3, pH 11.5 [to remove peripherally bound proteins (13)], yielded the same result. Thus, the porin is probably inserted into the outer membrane. However, treatment of the membranes with trypsin was sufficient to completely digest the monomer, whereas the trimer was only slightly digested (Fig. 5), a result supported by studies of trypsin proteolysis of outer membranes from either pop6510(pAC1) or pop6510 (pBsLAMB1). It has been shown previously that trimeric maltoporin remains resistant to trypsin even when solubilized in detergent, whereas monomeric maltoporin is readily digested (7,40). In our experiments (Fig. 5), the monomeric form of maltoporin in the outer membrane preparations was indeed completely digested by trypsin, whereas the trimer remained relatively resistant. This result was also confirmed by Western blotting using detection with anti-LamB polyclonal sera (Fig. 4).

DISCUSSION

The present study illustrates well the differences between the well-worked out strategies for the high-level expression of soluble proteins and the still poorly examined strategies for the overexpression of membrane (in this case, outer membrane) proteins in *E. coli*.

For water-soluble proteins, most strategies involve placing the target gene under control of a strong inducible promotor (e.g., λp_L, T7, *tac, trc*) on a high (pUC-derived, 200–500 copies/cell) or intermediate [pBR322-derived, pACYC184-derived (both 20–50 copies/cell)] copy number plasmid, and growing the

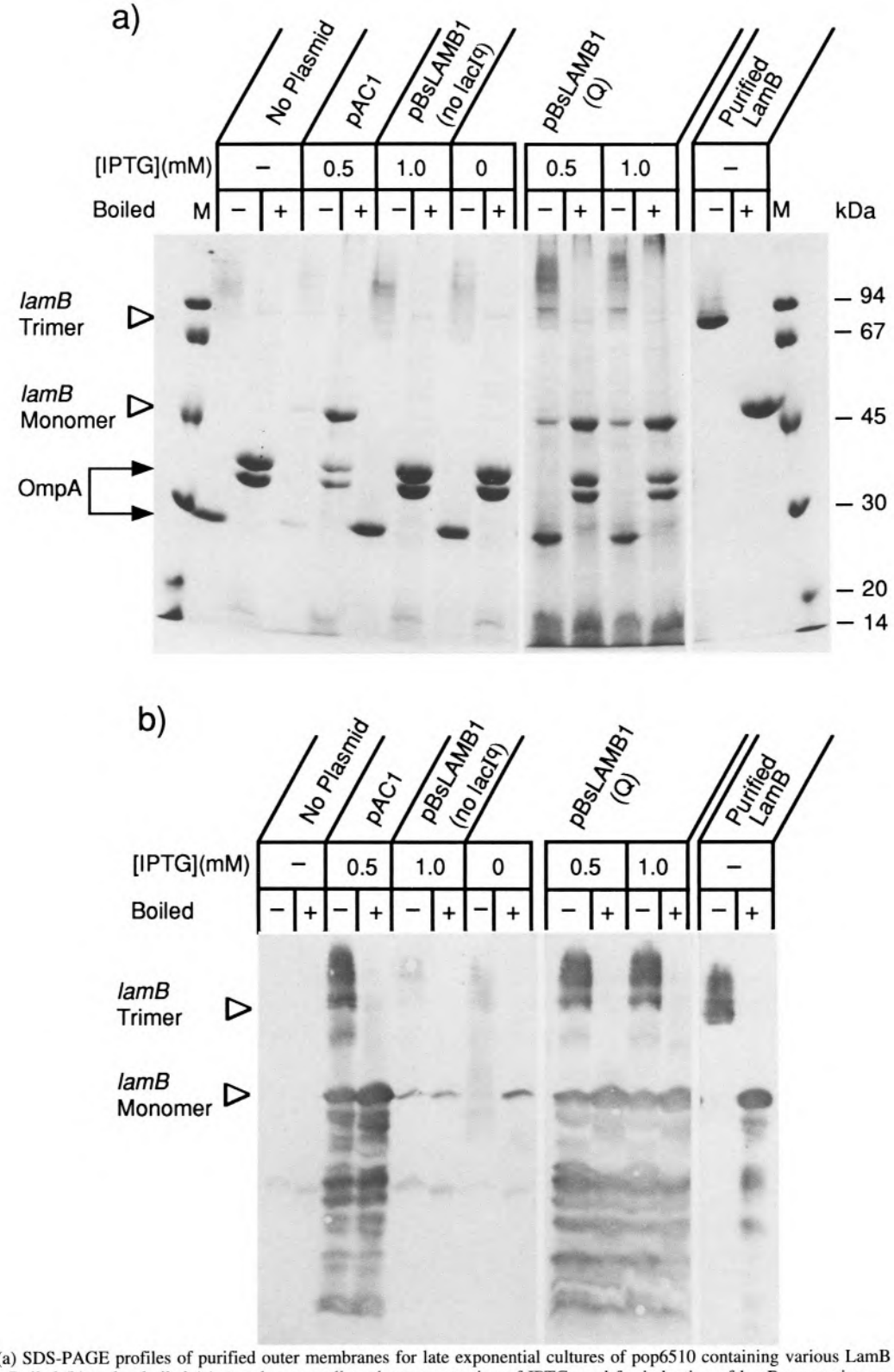

FIG. 4. (a) SDS-PAGE profiles of purified outer membranes for late exponential cultures of pop6510 containing various LamB expression plasmids. Boiled (b) and unboiled (u) samples, as well as the concentration of IPTG used for induction of lamB expression, are indicated. Samples of boiled and unboiled purified LamB are shown for comparison. (b) A Western blot using an anti-LamB polyclonal antisera of the samples shown in (a). Detection was performed using horseradish peroxidase. For both (a) and (b) the migration of trimeric and monomeric LamB is indicated. Q indicates that expression was performed in the presence of pACYCLACIQ1. The large number of immunopositive bands running underneath the LamB monomer is due to proteolytic digestion of the protein by outer membrane or periplasmic proteases, probably occurring after the membrane has been solubilized in SDS. A nonspecific cross-reaction with cellular protein can be eliminated, as the strain pop6510 lacking recombinant LamB shows almost no signal (see first two lanes). Despite the strong immunopositive signal for the small proteolytic fragments in the Western blot, the corresponding lanes of the Coomassie Blue-stained gel show that they only represent a very small fraction of the total protein.

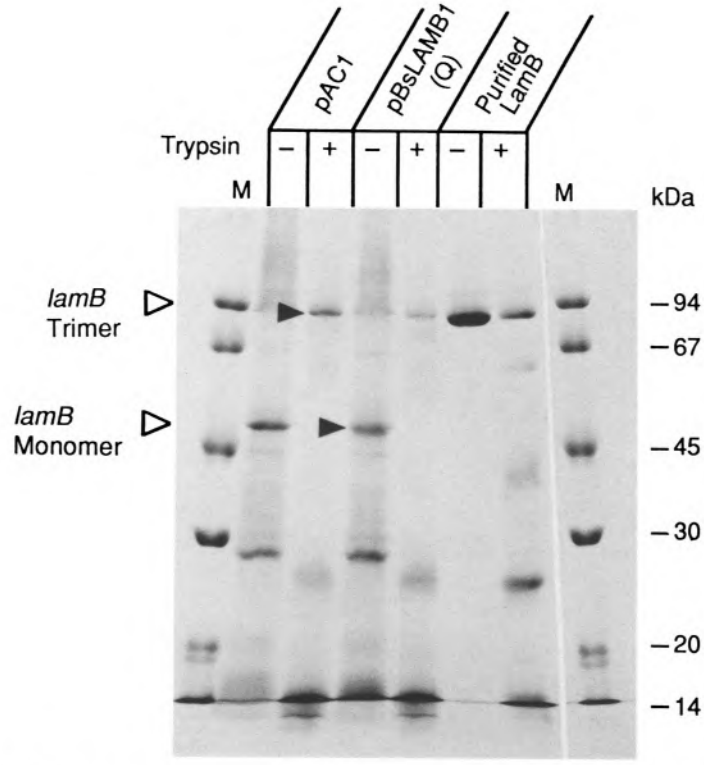

FIG. 5. SDS-PAGE profiles of purified outer membranes from IPTG-induced cultures of pop6510 (pAC1) and pop6510 (pBsLAMB1/pACYCLACIQ1) after treatment with trypsin (see Materials and Methods). A sample of purified LamB treated identically is shown for comparison. The expected migration of the LamB trimer and monomer is indicated.

cells without induction until the early or mid-exponential phase. After induction, cells are allowed to grow for 2–8 h (depending on the target gene and strain) and then harvested. The latter time interval minimizes the effects of toxicity and proteolysis that may occur. Implicit in these strategies are two assumptions: (a) the amount of available space (the cytoplasm) is not limiting; and (b) the capacity of the processing machinery (e.g., chaperones, N-terminal processing soluble components) are largely not limiting.

Particularly for outer membrane proteins, assumptions (a) and (b) may not be valid. First, it has been shown previously that the total amount of protein that can assemble in the outer membrane is regulated in some way by the cell (10,12,29,35) and that overexpression of one component reduces the expression of other outer membrane components. This implies that an upper limit exists to the amount of protein that can be expressed in the outer membrane. This observation also suggests that as yet unknown regulatory mechanisms are operating, independent of promoter strength, to adjust the amount of protein that can assemble in the outer membrane. The results presented here support these observations. For instance, although the copy numbers of pBsOMPF1 and pBsOMPC1 are 5- to 10-fold higher than that of the BR322-derived vectors pMY222 pand pMY150, respectively, only a twofold increase in the amount of product in the outer membrane is observed (Fig. 3, Table 2). In addition, increasing the amount of OmpF or OmpC causes a corresponding decrease in another major membrane protein, OmpA. These observations suggest that in the systems described above the limit of outer membrane protein expression has been reached. Both the *ompF* and *ompC* promoters, which can produce up to 10^5 copies/cell from a single chromosomal gene, are rather strong (10,23,38). Even using the very strong T7 promotor, no further increase in expression can be achieved (A. Prilipov and J. P. Rosenbusch, unpublished data).

The effect of limiting expression was also observed for the studies with PhoE porin. For the high copy number plasmid pBsPHO2, the amount of protein produced was only twofold that of pJP29, even though the promoter region (the *pho* box) remained unchanged (Table 2). Interestingly, even with pBsPHO2 (*lac* and *pho* promoters in tandem) the expression level of PhoE was roughly equivalent to that from pBsPHO1, and never attained that of OmpF or OmpC. We are unable to explain this difference at present.

The influence of limiting processing capacity has led us to employ a continuous expression strategy for

the *ompF, ompC*, and *phoE* genes. Several considerations are relevant: 1) Under appropriate environmental conditions, *E. coli* naturally expresses these porins at high level. This implies that the SecB chaperonins involved in binding the nascent pre-polypeptide and subsequent transport through the cytoplasmic membrane [see (34)] via the SecA/Y/E/G machinery are not limiting during growth. 2) The plasmids pMY222, pMY150, and pJP29 were previously shown to express their target genes constitutively (pJP29 requires the *phoS*$^-$/*phoR*$^-$ backgound CE1248 for constitutive expression) without noticeable toxic effects (4,29,36). 3) In the case of PhoE, the amount of protein obtainable with the strain CE1248 is consistently higher than that obtained from the inducible strain CE1224 (48). 4) During recent studies (19) aimed at generating 2D crystals in situ by high-level expression of recombinant porins in an *ompA*$^-$ strain, it was observed that the outer membrane "fills up" with the plasmid-encoded porin during the late exponential phase. This is consistent with the well-established rule that plasmid-driven expression continues as expression of chromosomal genes is tightly regulated in this phase. 5) Proteolysis of outer membrane proteins in situ is minimal. It has been estimated that the average lifetime of an outer membrane protein was approx. 72 h (42).

For the OmpF, OmpC, and PhoE porins, continuous expression appears to be successful, judging from the quantity of protein assembled in the outer membrane. As shown from the growth curves obtained for OmpF and OmpC (Fig. 1a, b) and SDS-PAGE profiles (Fig. 3) obtained for BZB1107(pBsOMPF1) and BZB1107(pBsOMPC1), expression of these outer membrane porins may have reached the expression limit of the outer membrane as growth rates of strains containing these plasmids are significantly slowed although the final cell densities achieved are comparable to the corresponding pBR322 derivatives. Cloning of the porins under constitutive promotors may be performed by the use of the strain RR28. The strain was originally selected because of its ease of transformation with large plasmids, and the efficiency of lysis, allowing high amounts of plasmid DNA to be isolated. Our results show that RR28 exhibits a low level of outer membrane porin expression, probably due to an undefined mutation in the *sec* translocation pathway, which obviates possible toxic effects of constitutive high-level expression.

On the other hand, expression of *lamB* does appear to conform to the observation that porins expressed with high copy number vectors are toxic. The addition of IPTG to pop6510(pAC1) causes an abrupt retardation of growth and only low final cell densities are attained. The comparison of the expression levels of *lamB* using pop6510 (pAC1, a pBR322-derived plasmid with a *tac* promotor) and pop6510 (pBsLAMB1/pACYCLACIQ1) (using the pBs-derived plasmid *lac* promotor) is interesting. The *tac* promotor is known to be about fivefold stronger than the *lac* promotor (1), and taking the different plasmid copy numbers into account, cells containing pBsLamB1 should yield about twice the level of LamB than that observed for pAC1. However, after induction with 0.5–1.0 mM IPTG, approximately equal amounts of trimeric protein are obtained (Fig. 4), indicating that in both cases the expression level of the recombinant protein is at the limit attainable for the outer membrane. The toxicity of high expression levels of LamB may arise from the different core topology of this porin (41).

The most surprising observation for the expression of LamB from either pAC1 or pBsLamB1 is the appearance of significant levels of monomeric protein, which cannot be removed by alkaline washes nor by extraction with 2% SDS (to extract contaminating inner membrane and possibly inclusion bodies). Based upon these data, it appears that monomeric LamB is incorporated into the outer membrane, even at very low levels of expression, and thus remains susceptible to proteolysis by added trypsin. This result eliminates the possibility that monomeric LamB is present as insoluble inclusion bodies within the periplasmic space.

The mechanism of porin trimerization in vivo is relatively unclear, but several recent reports indicate that, at least for OmpF, trimerization requires the presence of lipopolysaccharides, and may occur prior to insertion into the outer membrane (33,43). These studies show that even at high levels of overexpression of OmpF, PhoE, and OmpC, the insertion of monomers into the outer membrane does not occur significantly. In contrast, LamB monomer insertion is very frequent and independent of trimer formation and may indicate that LamB is more suited to biotechnological applications than the other porins studies here, as the insertion of foreign epitopes which prevent trimer formation will not be necessarily deleterious for expression and surface exposure.

The usefulness of the overexpression observed for the new pBs-derived vectors described here has been recently demonstrated in two cases. First, the twofold increase in expression observed for pBsPHO1 and pBsPHO2, compared to pJP29, led to a simplified extraction procedure for PhoE and more reproducible production of three-dimensional crystals diffracting to high resolution (11). Second, the expression levels observed for pBsPHO1 and pBsLAMB1 were sufficient to produce two-dimensional arrays in situ in the outer membrane of an OmpA-deficient strain and allowed visualization of the in-plane projection of PhoE and LamB by digital imaging techniques (19),

whereas the parent vectors (pJP29 and pAC1, respectively) failed to do so.

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