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MicroCorrespondence

Potential role of two *Helicobacter pylori* relaxases in DNA transfer?

Sir,

The recent determination of the complete *Helicobacter pylori* genome sequence (Tomb *et al*., 1997, *Nature* **388**: 539–547) and functional studies on the CAG pathogenicity island (Censini *et al*., 1997, *Proc Natl Acad Sci USA* **93**: 14648–14653; Covacci *et al*., 1997, *Trends Microbiol* **5**: 205–208) are major contributions to our understanding of this important pathogen. Current investigations focus on the CAG genes coding for a cytotoxin-associated antigen (CagA, ORF 547) and potential virulence factors such as homologues of *Agrobacterium* VirB4 (ORF 544), VirB7 (lipoprotein CagT, ORF 532), VirB9 (ORF 528), VirB10 (ORF 527), VirB11 (ORF 525) and VirD4 (ORF 524) proteins. Three additional *virb4* gene copies (ORFs 017, 441 and 459) as well as a gene encoding a vacuolating toxin (VacA, ORF 887) are located outside the CAG region. CagA, VacA and a few other proteins were found to be secreted and to enhance the inflammatory response in the gastric mucosa. The presence of *vir* genes and several GC-rich islands further suggests the existence of an adapted DNA-transfer apparatus for delivering virulence genes across bacterial boundaries. Evidence for conjugation-like DNA-transfer mechanisms between *Helicobacter pylori* strains has already been demonstrated *in vitro* (Kuipers *et al*., 1998, *J Bacteriol* **180**: 2901–2905), but genetic determinants remain unknown.

An intriguing aspect is the sequence homology of the CAG-encoded proteins to the membrane pore-forming VirB proteins of the *Agrobacterium* tumour-inducing (Ti) plasmid for interkingdom export of transfer (T)-DNA from bacteria to plant cells. On the basis of this homology, the CAG island was recently suggested to code for an ancient secretion apparatus that is capable of exporting a variety of proteinaceous material, and possibly also nucleoprotein particles, from *Helicobacter pylori* (Christie, 1997, *Trends Microbiol* **5**: 264–265). Each of the six proposed proteins in this system is also related to components forming the export machinery for the *Bordetella pertussis* toxin and broad-host-range DNA plasmids (Pansegrau and Lanka, 1996, *Prog Nucleic Acid Res Mol Biol* **54**: 197–251). Interestingly, members of the VirD4 protein family that have been suggested to link the T-DNA complex directly to the exporting membrane channel have to date been detected only in agrobacterial Ti-plasmid and conjugative plasmid DNA-transfer systems but not in protein transporters. Besides the VirD4 encoded in the CAG island, there is another putative VirD4 homologue (TraG, ORF 1006) present in the *Helicobacter pylori* sequence. Other VirD proteins representing essential components of a hypothetical DNA-transfer apparatus of *Helicobacter pylori* have not been identified so far. Likewise, a DNA-processing

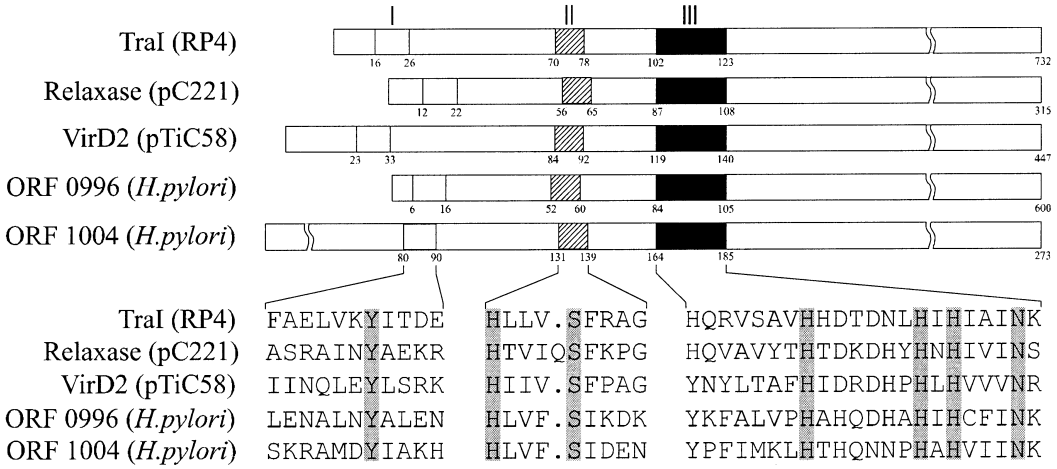

Fig. 1. Alignment of three conserved functional motifs (I–III) in DNA relaxases encoded in mobilizable plasmids of Gram-negative (RP4; accession no. Q00191) and Gram-positive (pC221; accession no. A04494) bacteria, in *Agrobacterium* Ti-plasmids (pTiC58; accession no. M33673) and ORFs 996 (accession no. D64644) and 1004 (accession no. D64645) from *Helicobacter pylori*. Identical amino acids are shaded with dark grey and at least three identical or similar functional residues with grey. Motif boundaries are given by the corresponding number of amino acid positions.

machinery including relaxase VirD2 as a second major component would be essential for the specific cleavage and binding of the T-DNA.

By searching the *Helicobacter pylori* genomic sequence in the TIGR database for potential homologues of VirD2, we found two ORFs in a third GC-rich island downstream of the CAG region with significant homology to relaxases. Figure 1 shows the three conserved functional domains between *Helicobacter* ORFs 996 and 1004, relaxases from *Agrobacterium* Ti-plasmids and relaxases from mobilizable plasmids of Gram-negative and Gram-positive bacteria. These motifs are usually located at the N-terminus of the proteins and are arranged in a specific order (Pansegrau and Lanka, 1996, *Prog Nucleic Acid Res Mol Biol* **54**: 197–251). Two regions designated as motifs I and III are involved in catalysing the nicking–closing reaction at specific DNA target sequences. Motif I carries the tyrosine residues that covalently attach the relaxase in a transesterification reaction to the 5′ terminus of the cleaved single-stranded T-DNA. Motif II is thought to function as a DNA recognition domain. Motif III contains an HUH sequence pattern essential for relaxase activity and is therefore believed to activate the aromatic hydroxyl group of tyrosine in motif 2 by proton abstraction.

The detection of two ORFs with significant similarity to conjugative and T-DNA transfer systems strongly supports the notion of a DNA-transfer apparatus in *Helicobacter pylori*. The relaxases might act in concert with the VirD4 and VirB proteins of the proposed membrane pore described above. Whether the relaxases participate in normal bacterial conjugation processes and/or the transfer of DNA to infected target cells, e.g. gastric epithelial cells, remains to be elucidated in future studies.

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Potential receptor function of three homologous components, TatA, TatB and TatE, of the twin-arginine signal sequence-dependent metalloenzyme translocation pathway in *Escherichia coli*

Sir,

It is well established that plant thylakoids possess a Sec-independent, ΔpH-driven import pathway for which the substrates are synthesized with cleavable targeting signals with an essential twin-arginine motif (reviewed by Robinson and Mant, 1997, *Trends Plant Sci* **2**: 431–437). Growing evidence strengthens the idea that a similar mechanism is used in the targeting and translocation of a subset of bacterial metalloenzymes across the cytoplasmic membrane (Bogsch *et al*., 1998, *J Biol Chem* **273**: 18003–18006; Santini *et al*., 1998, *EMBO J* **17**: 101–112; Sargent *et al*., 1998, *EMBO J* **17**: 3640–3650; Weiner *et al*., 1998, *Cell* **93**: 93–101). A component of this pathway, HCF106, was first identified in maize (Settles *et al*., 1997, *Science* **278**: 1467–1470). Database searches with the HCF106 sequence revealed homology to open reading frames from all completely sequenced bacterial genomes, and related sequences are found in organisms ranging from archaebacteria to higher eukaryotes, suggesting that the twin-arginine pathway is a ubiquitous system for membrane targeting and translocation of a subset of proteins (Settles *et al*., 1997, ibid; Weiner, 1998, ibid).

HCF106 contains 243 amino acids with a predicted membrane-spanning domain (MSD) from amino acid 68 to 89. The conserved region is restricted to the MSD and approximately 40 residues of the adjacent C-terminal domain, which is predicted to form an amphipathic alpha helix. The two *Escherichia coli* homologues of HCF106 are located in *yigTUW* (86 min) and *ybeC* (14 min) loci (Settles *et al*., 1997, ibid). The first sequence revision of the *yig* region assigned three genes *mttABC* (for membrane targeting and translocation, corresponding to *yigTUW* with several sequence corrections in *yigT*) to this locus (Weiner, 1998, ibid). The MttA protein was predicted to contain 277 amino acids with two MSDs and a long C-terminal periplasmic domain. The reported HCF106 homologous region comprises 43 amino acids from residues 17 to 59, including the first MSD. Substitution of proline 128 of MttA by leucine (P128L) results in cytoplasmic accumulation of the catalytic subunit DmsA of the membrane-bound DMSO reductase (Weiner *et al*., 1998, ibid). Likewise, active TMAO reductase TorA accumulated in the cytoplasm in the mutant strain, but the normally periplasmic nitrate reductase NapA become associated with the membrane fraction. Weiner *et al*. proposed that the periplasmic domain of MttA might play a role in gating the pore, with MttB protein as the pore for protein transport and MttC as a receptor function for twin-arginine proteins.

A recent revision of the DNA sequence and gene product identification revealed the presence of a stop codon in the *mttA* gene (Sargent *et al*., 1998, ibid). The *mttA* gene is thus divided into *tatA* and *tatB* (for twin-arginine translocation). TatA is composed of 89 amino acids corresponding to the region from the methionine 14 to the valine 103 of MttA. The C-terminal 171 residues of MttA was renamed TatB. The new nomenclatures for *mttB* (*yigU*), *mttC* (*yigW*) and *ybeC* are *tatC*, *tatD* and *tatE* respectively (Table 1). TatA and TatE share about 50% sequence

Table 1.Components of the TAT pathway and their mutation effects on different metallo-enzymes.

Locus	New nomenclature	Effects of mutation on the translocation of [a]					
		Hyd2	Hyd1	TorA	DMSO	Nap	FDH-N
ybeC (14 min)	*tatE*	+/−[1]	+[1]	−[1]	+/−[1]	?	+/−[b1]
yigT (86 min)	*tatA*	−[1]	−[1]	−[1]	−[1]	?	−[b1]
	tatA–tatE	−[1]	− −[1]	− −[1]	−[1]	?	− −[b1]
yigT (mttA)	*tatB*	+[2]	?	− −[2,3]	− −[3]	− −[c3]	?
yigU (mttB)	*tatC*	− −[4]	− −[4]	− −[4]	− −[4]	?	− −[b4]
yigW (mttC)	*tatD*	?	?	?	?	?	?

a. The translocation of hydrogenase 2 (Hyd2) and 1 (Hyd1), TMAO reductase (TorA), DMSO reductase (DMSO), nitrate reductase (Nap) and formate dehydrogenase (FDH-N) was not (+), slightly (+/−), partially (−) or completely (− −) blocked by mutation in the corresponding genes on the left. The results are described in (1) Sargent *et al*., 1998, ibid;(2) this study; (3) Weiner *et al*., 1998, ibid; (4) Bogsch *et al*., 1998, ibid.
b. Activity was also affected.
c. Associated with the membrane fraction.

identity at the protein level. Disruption of *tatA*, *tatE* and *tatC* results in different effects depending on the enzymes analysed. The effects of *tat* mutations on seven enzymes known to use this pathway are summarized in Table 1.

We cloned *tatABCD* and constructed a *tatB* mutant by the insertion of a Kan^R cassette at the *Sac*II site, which interrupts the TatB at amino acid position 88. The downstream region of the Kan^R cassette does not have a transcription terminator but contains an optimal ribosome binding site and, thus, does not have a polar effect on the expression of *tatCD*. Like the *mttA* P128L mutation, *tatB*::Kan^R results in the accumulation of active TMAO reductase in the cytoplasm. In addition, no precursor of this enzyme is translocated into the periplasm, which is consistent with our previous observation that only the cofactor-containing active forms are competent for translocation (Rodrigue *et al*., 1996, *FEBS Lett* **392**: 81–86; Santini *et al*., 1998, ibid). Surprisingly, the interruption of *tatB* has no effect on the membrane targeting and translocation of hydrogenase 2 (HYD2), which does not support the idea that *mttA* is involved in the translocation of all twin-arginine signal-bearing redox enzymes.

As the reported region of homology between YigT and HCF106 is located in TatA, it was assumed that TatB would be unrelated to HCF106 (Sargent *et al*., 1998, ibid). The phenotypes of the *mttA* and *tatB*::Kan^R mutants suggest, however, that TatB is another counterpart of TatA and TatE and is involved in the translocation of a subset of twin-arginine redox enzymes. To test this idea, we first analysed the local similarity between HCF106 and TatE, TatA and TatB by using the SIM program (Huang and Miller, 1991, *Adv Appl Methods* **12**: 337–357). To facilitate direct comparison, the three Tat proteins were artificially fused together and compared as a single polypeptide with HCF106. Three regions, corresponding to the N-termini of TatE, TatA and TatB, show the same similarity score with the conserved region of HCF106 (Fig. 1). In addition, a segment covering the amino acids from 170 to 220 shares some similarity with the C-terminal region of TatB (data not shown). We then analysed the statistical significance of the similarities between HCF106 and the three Tat proteins using the RDF2 program (Pearson and Lipman, 1988, *Proc Natl Acad Sci USA* **85**: 2444–2448). The comparison score between HCF106 and TatB was as good as that between HCF106 and TatE (Fig 1, 8.1 vs. 8.4). The probability of getting such a score by chance is less than 10^{-16}. These results confirm that HCF106 is homologous to all three Tat proteins and strongly support the idea that they have a common evolutionary origin.

The cellular location of TatA, TatB and TatE was predicted according to Boyd *et al*. (1998, *Prot Sci* **7**: 201–205) and von Heijne (1992, *J Mol Biol* **225**: 487–494). Their MaxH values are significantly higher than the discriminator value of 1.53 (Fig. 1) and allow us to assign them unambiguously as membrane proteins. Furthermore, their C-terminal regions are most likely to be at the cytoplasmic side of the membrane. Therefore, TatA, TatB and TatE seem to function, independently or together as a trimer, as membrane receptors for different subsets of redox proteins, each one showing different affinities for the

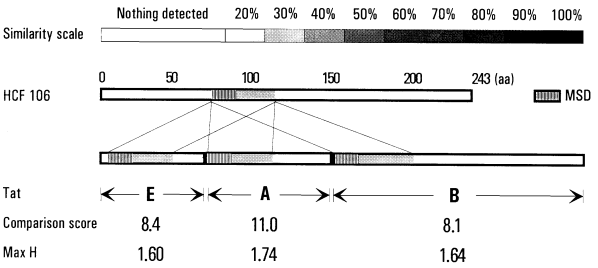

Fig. 1. Local similarity was analysed using BLOSUM62 as comparison matrix, and 20 alignments were computed. The gap open penalty and gap extension penalty were 12 and 4 respectively. RDF2 comparison scores were obtained by 500 local random shuffles (window size of 10) of the three entire Tat proteins with a ktup of 2 against Hcf106. MaxH values were calculated with a window size of 17. MSD, membrane-spanning domain.

substrates, which accounts for the effects of the same mutation on various enzymes. The heterotrimer model would further explain the observation that interruption of any one of the three genes severely affects the translocation of TorA. These three branches of the TAT pathway would join together at the TatC channel level, as the *tatC* mutation affects the translocation of all enzymes analysed (Table 1). The amphipathic alpha-helix domain in the conserved region might be the best candidate for the site at which these receptors interact with each other and/or with TatC. The *tatD* (*yigW*, *mttC*) gene encodes a soluble protein that shows sequence homology with the products of *E. coli* ORFs *yjjV* and *ycfH* and whose function has not been assigned experimentally (Sargent *et al*., 1998, ibid; Weiner *et al*., 1998, ibid). The MaxH value of 1.36 and the absence of a signal sequence from TatD suggests that this potential Tat component is cytoplasmic and could play a role in the early stage of protein translocation. Taken together, these results suggest that the homologous components TatA, TatB and TatE might constitute three branches at the membrane targeting/reception stage of the TAT pathway and that the three different branches join together at the channel level.

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Importance of using *lac* rather than *ara* promoter vectors for modulating the levels of toxic gene products in *Escherichia coli*

Sir,

Soon after the advent of gene cloning, plasmid expression vectors were developed in which variants of the *lac* promoter were used to direct high level IPTG- (or lactose-) inducible synthesis of recombinant proteins in *Escherichia coli* (Brosius, 1984, *Gene* **27**: 161–172; Amann and Brosius, 1985, *Gene* **40**: 183–190). Typically, the products of genes cloned for expression in such vectors (e.g. P_{TAC} and P_{TRC} vectors) account for 5–30% of the total cell protein after induction. However, such high-level synthesis often results in inclusion body formation, and this prevents phenotypic analysis and, in some cases, isolation of biologically functional product. Moreover, with toxic products (for example many membrane proteins), it often proves impossible to subclone the gene into these vectors because the basal level of expression is sufficiently high that the growth of the host cells is severely compromised even in the absence of inducer (Grisshammer and Tate, 1995, *Q Rev Biophys* **28**: 315–422.

Recently, arabinose-inducible expression vectors were described that use the *araBAD* promoter (Cagnon *et al*. 1991, *Prot Eng* **4**: 843–847; Guzman *et al*. 1995, *J Bacteriol* **177**: 4121–4130). The pBAD vectors (Guzman *et al*. 1995, ibid.) have an inherently low basal level of expression (particularly in the presence of glucose, which causes catabolite repression) and hence they facilitate the successful subcloning of toxic genes. Induction by arabinose is rapid and results in 100- to 1000-fold increase in the rate of synthesis of the target protein, and good, but not massive, yields of it. The low basal levels and high inducible levels of gene products have led to the use of pBAD vectors both for depleting *E. coli* of specific gene products (in mutant hosts lacking the corresponding gene) and for overexpression studies when it is preferable to keep the protein soluble (Guzman *et al*. 1995, ibid.).

It has also been shown that by incubating cultures with increasing submaximal (non-saturating) concentrations of arabinose, the yields of the target protein can be reproducibly increased over a greater than 100-fold range, and this finding forms the basis for advocating the use of *ara* promoter vectors in experiments in which fine control over the levels of a toxic, or marginally soluble, product needs to be imposed (Guzman *et al*. 1995, ibid.; Invitrogen catalogue, 1998). However, experiments in which the synthesis of green fluorescent protein (GFP) was induced with non-saturating concentrations of arabinose have revealed that, although the final yield of GFP within cultures increases as inducer concentration is increased, the levels of GFP in different individual cells within a culture vary considerably, from high to intermediate to no detectable GFP. Moreover, within different populations both the proportion of cells making detectable GFP and the maximum level of GFP present in individual cells increased with increasing concentrations of arabinose (Siegele and Hu, 1997, *Proc Natl Acad Sci USA* **94**: 8168–8172). In fact, this pattern of partial induction is expected to apply to any control circuit in which expression of the gene encoding the uptake system for the inducer is itself subject to induction. Indeed, over 40 years ago an analogous autocatalytic induction phenomenon was seen when the *lac* operon was subjected to induction with submaximal levels of its natural inducer, lactose (Novick and Weiner, 1957, *Proc Natl Acad Sci USA* **43**: 553–566). Lactose is dependent upon the presence of the lac permease in the envelope for its

uptake into cells, and when the permease is present it actively transports the inducer into the cytoplasm. But the levels of permease present in the envelope of uninduced cells vary from none to a few molecules, depending on the recent history of the cell (i.e. when its *lac* operon last underwent a burst of transcription). Hence, when limited amounts of lactose are added to the medium only some of the cells will be capable of accumulating it, and in these cells the consequence of its uptake is further synthesis of the permease and thus more rapid uptake of any remaining lactose and, subsequently, full induction of the *lac* operon.

It is important to appreciate that after exposure to low levels of autocatalytic inducers the intermediate levels of gene product seen within a cell population belie a wide variation in levels of product from cell to cell. But when working with toxic or marginally soluble gene products and, indeed, with various other gene products that display more subtle overproduction phenotypes it can be crucial to ensure a low-to-intermediate level of expression in all cells. For example, many membrane proteins fail to assemble efficiently into the *E. coli* cytoplasmic membrane when highly overproduced (Ito and Akiyama, 1991, *Mol Microbiol* **5**: 2243–2253). Autocatalytic induction with limiting inducer would result in high-level synthesis in some cells and failure of a proportion of the membrane protein molecules therein to assemble into the membrane. In contrast, low-to-moderate rates of synthesis in every cell would be required to ensure membrane assembly of all the protein molecules synthesized.

Unlike lactose, the gratuitous inducer, IPTG, is not dependent upon the lac permease for its uptake into *E. coli* cells. Provided a permease-deficient, *lacY*, host is used, IPTG will enter the cytoplasm in a concentration-dependent manner. Hence, partial induction of the *lac* promoter, using IPTG in *lacY* hosts, can be used to fine-tune the levels of toxic gene products within individual cells, and not just within the population. Although early *lac* promoter-based expression vectors were inappropriate for the controlled expression of toxic genes (because they directed relatively high basal levels of expression) this problem can be overcome by positioning the *lac* promoter at a site in the vector where there is minimal readthrough transcription and by overexpressing the *lacI* gene (encoding the lac repressor) in the host cells. Hence, we propose that appropriately modified *lac* promoter vectors should be used (in conjunction with IPTG induction in *lacY* hosts) when it is important to synthesize low or intermediate levels of a particular protein within every cell. *Ara* promoter vectors will remain inappropriate until either a host strain is made that synthesizes the arabinose transporters constitutively or a gratuitous inducer is discovered that does not use/require them.

We have developed expression vectors in which the

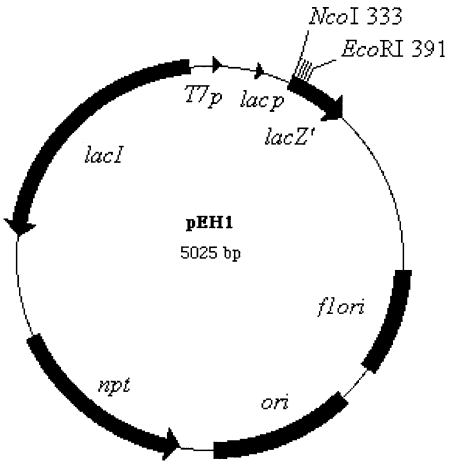

Fig. 1. Salient features of the *lacUV5* promoter, direct expression vector, pEH1. Further information on the kanamycin-resistant expression vector, pEH1, and its chloramphenicol-resistant analogue, pEH3, is provided in the text. pEH1 contains the following unique restriction sites in lacZ′: *Nco*I, *Bam*HI, *Hin*dIII, *Xba*I, *Kpn*I, *Sac*I, *Sma*I and *Eco*RI. Abbreviations: *lac p*, *lacUV5* promoter; *T7 p*, T7*lac* promoter; *lacZ′*, LacZ α-peptide coding region; *f1 ori*, phage f1 origin for single-stranded DNA replication; *ori*, pMB1 replication origin; *npt*, kanamycin resistance gene encoding neomycin phosphotransferase; *lacI*, lac-repressor gene.

lacUV5 promoter directs a low basal level of expression and a high (but not massive) level of IPTG-inducible expression (see Fig. 1). As we favour the direct expression approach, we have incorporated a ribosome binding site and modified *lacZ′* allele (with a unique *Nco*I site spanning its initiation codon, and further unique restriction sites within *lacZ′*), downstream of the *lacUV5* promoter, in both the kanamycin-resistant plasmid, pEH1, and its chloramphenicol-resistant analogue, pEH3. Therefore, coding regions from any organism can be subcloned for IPTG-inducible expression in *E. coli* (and transformants containing the recombinant expression plasmids can be readily identified using α-complementation). pEH1 and pEH3 also code for constitutive synthesis of the lac repressor, ensuring a low basal level of synthesis of the cloned gene product in any *E. coli* host strain, not just in *lacI*^{q} strains. In addition, they contain a T7*lac* promoter upstream of the *lacUV5* promoter. By supplying T7 RNA polymerase *in trans* the yield obtained from full induction with IPTG can be boosted, and (in the presence of rifampicin) the cloned gene product can be metabolically labelled (Studier *et al*., 1990, *Methods Enzymol* **185**: 60–89). Using a pair of vectors, with different selectable markers, facilitates rapid subcloning, expression and engineering of target genes. Moreover, as antibodies are commercially available against the kanamycin resistance gene product (neomycin phosphotransferase) and the chloramphenicol resistance gene product (chloramphenicol acetyl transferase), these

proteins can serve as internal standards in expression studies or as markers for cytoplasmic localization in analyses of membrane protein topology and protein export.

We have subcloned the regions encoding various toxic bacterial and eukaryotic membrane proteins (for example integral membrane proteins such as the mannitol permease of *E. coli*, and the human β_2-adrenergic receptor) into pEH1 and pEH3 for direct, IPTG-inducible, bacterial expression. *LacY* host cells carrying such plasmids produce low but detectable levels of these membrane proteins, and grow and form colonies normally. However, colony formation is completely prevented by the inclusion of submaximal concentrations of IPTG in L agar plates (in the case of the mannitol permease, the size of colonies is progressively reduced by the inclusion of 50 and 100 μM IPTG, respectively, and their formation is completely prevented by the inclusion of 250 μM IPTG). Finally, as with pBAD vectors, globular proteins and domains thereof (for example the DNA binding domain of the human Pax-5 transcription factor; personal communication, Dr B. Adams) can be produced in good yields and in a soluble and active form after full IPTG induction of the T7*lac* and/or *lacUV5* promoters of these pEH vectors.

The DNA sequences of pEH1 and pEH3 have been submitted to the EMBL database under the accession numbers AJ007659 and AJ007660, respectively. pEH1 and pEH3 are available from the authors on request.

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