[16] Cloning Outer Membrane Protein Genes and Studying Structure–Function Relationships

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Introduction

The cloning and expression of outer membrane proteins has often been problematic due to the high protein content of the bacterial outer membrane (1) and thus its finite capacity to accommodate new proteins. Another complication is that many outer membrane proteins of interest are expressed in their native background in high copy numbers (up to 200,000 copies per cell), signifying the high efficiency of their promoters. Thus, the preferred methods of cloning for most other genes, utilizing high copy number vectors, must often be avoided due to the potential for overexpression lethality. Even in foreign hosts the level of expression from many outer membrane protein gene promoters is so high that only low gene dosages (i.e., low copy number vectors) can be utilized. In this brief overview we describe what we perceive as the optimal procedures for cloning and manipulation of such genes.

Cloning of Outer Membrane Protein Genes

As mentioned above, the use of low or medium copy number plasmids as cloning vectors helps to control problems of overexpression lethality. Table I lists the copy number of some generally used, broad host range plasmids. Most outer membrane protein genes can be cloned on large low copy number plasmids or cosmids (2). However, in some cases, especially when being cloned into the homologous or a related host bacterium, this host might not even be able to tolerate a gene duplication. In these cases the use of a lysogenic phage (e.g., λ-based) vector (commonly used to make genomic libraries) is the best option for primary cloning. This has the added advantage, due to the large inserts tolerated, of limiting the amount of screening, an important factor given the usual lack of selectable phenotypes for outer membrane proteins. If a fairly long probe is available [e.g., a polymerase chain reaction (PCR) fragment, a related gene from another species, or flanking DNA from a transposon-inactivated gene] the gene may be cloned (or subcloned) as two biologically inactive fragments as done for *Haemophilus influenzae* protein P2 (3). Conditions that yield two overlapping, hybridizing restriction fragments can be determined by constructing a restriction map made from Southern blots of chromo-

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TABLE I Cloning and Expression Vectors Suitable for Outer Membrane Proteins[a]

Plasmid	Copy No.[b]	Origin of replication	*oriT*[c] *(mob)*	Promoters or features[d]	Ref.
pRK404	>10	IncQ	Yes	P_{lac}; X-Gal	*e*
pRK415(767)	>10	IncQ	Yes	P_{lac}; X-Gal	*f*
pUCP18/19	~100[g]/~15[h]	ColE1/RP1[i]	Yes	P_{lac}; X-Gal	*j*
pMMB66HE	>10	IncQ	Yes	P_{lac}; *lacI*^{q}; X-Gal	*k*
pVDtac29	>10	IncQ	Yes	P_{lac}; *lacI*^{q}; X-Gal	*l*
pNM185	>10	IncQ	Yes	P_{tol}[m]	*n*
pLAFR	1-4	IncP	Yes	Cosmid	*o*
pDOC55	~20	ColE1 (pMB1)	—[p]	P_{lac} (antisense) λP_L(sense)	*q*

[a] For more details and possibilities, consult the references below and Ref. (32) in text.
[b] Copy number in *E. coli* (based on the origin of replication).
[c] Allows plasmid to be mobilized from *E. coli* S17-1 (29) or via triparental mating.
[d] X-Gal refers to the capacity for α-complementation of *lacZ* ΔM15 *E. coli* mutants so that the blue/white screen for recombinant plasmids can be employed.
[e] G. Ditta, T. Schmidhauser, E. Yakobson, P. Lu, X.-U. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski, *Plasmid*. **13,** 149 (1985).
[f] N. T. Keen, S. Tamaki, D. Kobayashi, and D. Trollinger, *Gene* **70,** 191 (1988).
[g] In *E. coli*.
[h] In *P. aeruginosa*.
[i] Contains a 1.8-kb "stabilizing" fragment that allows replication in various gram-negative bacteria.
[j] H. P. Schweizer, *Gene* **97,** 109 (1991).
[k] V. Morales, M. M. Bagdasarian, and M. Bagdsarian, *in* "Pseudomonas: Biotransformations, Pathogenesis, and Evolving Biotechnology" (S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan, eds.), p. 229. American Society for Microbiology, Washington, D.C., 1990.
[l] V. Deretic, S. Chandrasekharappa, J. F. Dill, D. K. Chatterjee, and A. M. Chakrabarty, *Gene* **57,** 61 (1987).
[m] Toluene-inducible promoter provides strong expression in *P. aeruginosa*.
[n] N. Mermod, J. L. Ramos, P. R. Lehrbach, and K. N. Timmis, *J. Bacteriol.* **167,** 447 (1986).
[o] N. T. Keen, S. Tamaki, D. Kobayashi, and D. Trollinger, *Gene* **70,** 191 (1988).
[p] Not broad host range.
[q] C. D. O'Conner and K. N. Timmis, *J. Bacteriol.* **169,** 4457 (1987).

somal DNA digests. Gel-purified DNA of the proper size can be cloned and later religated after modification of the genetic control sequences (see below).

Subcloning a smaller fragment onto a plasmid to permit subsequent manipulation of the sequences is advantageous. However, the increase in gene dosage associated with this is often intolerable to the host cell given that most common small vectors have high copy numbers. Therefore, inactivation or alteration of the cloned gene's promoter may be necessary. This can be accomplished by site-directed mutagenesis of a small restriction fragment containing the promoter and amino terminus of the gene followed by reunification of the complete coding region (4). Alternatively, PCR can be employed to amplify just the coding region of the gene, which could then be cloned behind a tightly regulated promoter. In our hands the use of strains

deficient in most major outer membrane proteins [e.g., CE1248 (5)] also permits higher expression of a foreign outer membrane protein.

A useful method for screening a genomic library for clones containing an outer membrane protein gene is the use of antibodies directed against outer membrane proteins on colony immunoblots (6). However, in the primary screening of the gene, expression levels must be sufficient to allow binding of the antibodies. A foreign gene cloned into *Escherichia coli* might express poorly or not at all due to a promoter that is unrecognized in the foreign genetic background. In our experience this is common for positively regulated genes. If the levels of the gene's expression are not adequate, or antibodies are not available, it is advisable to obtain an amino-terminal sequence of the protein in question (7). (This sequence is usually necessary in any event to confirm the cloning of the proper gene.) When a sequence is obtained, degenerate oligonucleotides can then be used as a tool to assist in the cloning of the gene. However, screening with oligonucleotides is technically difficult, especially if low copy number vectors are used (8). Therefore, decreasing the number of colonies to screen with oligonucleotides is advisable. For example, the oligonucleotides can be used to probe Southern blots of restriction-digested chromosomal DNA and permit definition of a size-fractionated subset of the genome for use in the construction of a mini-library. This was done for the *oprD* gene of *Pseudomonas aeruginosa* and is described in detail below. Alternatively, PCR can be employed in order to make an oligonucleotide that binds in a more stringent manner. Two degenerate oligonucleotides with opposite orientations from opposite ends of the known protein N-terminal sequence can be used with a chromosomal template to create a PCR product with 100% homology in the region between the oligonucleotides. Subsequent cloning of the PCR product will create a probe that is nondegenerate over 60 nucleotides (if 20 amino acids are obtained), rather than a pool of degenerate 15 to 21-mers, thus vastly simplifying and increasing the sensitivity of screening by colony blot hybridization.

For obtaining an amino-terminal sequence, the high copy number of an outer membrane protein is advantageous since it permits simple purification. We have routinely utilized the technique of Matsudaira (9) which involves transfer of proteins to poly(vinylidene difluoride) Immobilon membranes (Millipore Corp., Bedford, MA) for direct sequencing. However, in several cases we and others have observed blocked N termini, necessitating the use of chemical or proteolytic peptidation to permit sequencing. A general approach to primary cloning of outer membrane protein genes can be briefly described by the following example of the cloning of the gene for outer membrane protein D2 (*oprD*) of *P. aeruginosa* and expression in *E. coli* CE1248 (7, 5).

OprD was partially purified from *P. aeruginosa* PA01 and the N terminus of the protein sequenced as follows: D A F V S D Q A E A K G F I E D S. Taking into account codon bias in *P. aeruginosa*, a corresponding 29-mer oligonucleotide was deduced from amino acids 6–15 (although we now recommend the use of the PCR

method described above). This was then radiolabelled with [γ-^{32}P]ATP and used as a probe in Southern hybridization analysis with *P. aeruginosa* chromosomal DNA that had been singly and pairwise digested with several restriction enzymes. The location, but not the direction of transcription, of the N terminus of the *oprD* gene was mapped between a *Bam*HI and an *Eco*RI site. Since the apparent molecular weight of OprD on sodium dodecyl sulfate–poly acrylamide gel electrophoresis (SDS–PAGE) is 47,000, the approximate size of the *oprD* gene would be about 1.3 kilobases (kb). Therefore, fragments were isolated which should contain the whole *oprD* gene regardless of the direction of gene expression. These fragments were then used to create minilibraries from the isolated subsets of the chromosomal DNA with a suitable vector. The γ-^{32}P-labeled N-terminal-specific oligonucleotide was used as a probe to screen colonies from each library. Positive colonies were analyzed by restriction mapping and representatives of both orientations of the cloned fragment (with respect to plasmid promoters) were used for further analysis.

Expression studies with the various subclones were performed in *E. coli* CE1248 (5), a mutant which lacks the major *E. coli* porins OmpF, OmpC, and PhoE. *Escherichia coli* CE1248(pBK19), which had a 2.1-kb *Bam*HI–*Kpn*I fragment cloned in the same orientation as the *lac* promoter, revealed high expression of OprD in the *E. coli* outer membrane to a level almost equivalent to that of the *E. coli* major outer membrane protein OmpA. In contrast, in *E. coli* CE1248(pBK18R), which had the same fragment cloned in the reverse orientation to the *lac* promoter, only weak expression was observed. This indicated that the cloned *Bam*HI/*Kpn*I fragment contains the *P. aeruginosa oprD* gene promoter that can be recognized by *E. coli* but that OprD is weakly expressed from its own promoter in this genetic environment.

Another useful method for the cloning of a gene is to tag the DNA within the structural gene with a selectable marker. The use of transposons on suicide vectors to elicit a deficient phenotype is a common tagging method [e.g., pMT1000 (10)]. The tag, however, can theoretically reside in the gene, its operon (if polar), or its regulon. Since elements of regulons are not required to be physically linked on the chromosome (they may be coding for or controlled by *trans*-acting elements), the tag may turn out to be useful for defining the regulation of an outer membrane protein (7) but not for cloning the structural gene of the protein in question. If the gene is known to be part of a regulon, such as the Pho regulon, then a screen for normally controlled expression of other elements of the regulon will help in elucidating the likelihood of the tag being found in the structural gene. By using the selectable marker of the transposon, the transposon and flanking chromosomal DNA can be easily cloned from the mutant strain. The excised flanking DNA can then be used as a probe for the cloning of the gene from the chromosome of the parent strain. Transposon Tn*501*, for example, contains *Eco*RI sites 15 base pairs from either terminus and has no *Pst*I sites, providing a clonable *Pst*I fragment with *Eco*RI–*Pst*I liberated probes (11, 12).

The regulatory systems of bacteria may or may not be conserved between species.

This lack of conservation could cause either unregulated expression or no observable gene product if a repressor or activator is, respectively, utilized in gene regulation. Many genetic control elements appear to have diverged early in bacterial evolution and this permits the regulatory DNA sequences found on cloned genes to be controlled by host elements (13, 14). If a given gene is inducible in its native genetic background, then providing similar inducible growth conditions for the cloning host might allow expression of the normally regulated promoter of a cloned gene. OprP from *P. aeruginosa*, for example, is controlled by the Pho regulon in *E. coli* (13). Alternatively, poorly expressed genes can be cloned behind any one of a number of regulated promoters on multicopy plasmids (15). Some systems allow tight regulation of their promoters. For example, the system described by O'Conner and Timmis (16) employs antisense promoters to avoid expression due to the leakiness sometimes displayed by normally regulated promoters when cloned on multicopy plasmids. It is also possible to use background strains that overproduce repressor [e.g., DH5αF'IQ (Bethesda Research Laboratories, Gaithersburg, MD)], thus reducing expression from a promoter whose normal cellular supply of repressor can be titrated out by multiple copies of a derepressible promoter.

Table I shows some useful constructs that have the advantage of being broad host range, so that the cloned gene and its constructed variants can often be returned to the original species for assay. Methods for specifically inactivating the chromosomal copy of the gene are described in the next section.

Insertion Mutagenesis of Bacterial Outer Membrane Protein Genes for Functional Studies

The function of outer membrane proteins can be investigated by inactivating the gene and examining the effects of this knockout mutation on function. Chromosomal genes for outer membrane proteins can be inactivated in two ways. These involve direct transposon mutagenesis of the gene in the host bacterium or indirect insertional mutagenesis of the cloned gene in *E. coli* followed by gene replacement in the host bacterium. The former approach appears at first glance to be simpler but suffers from a major drawback, namely, the lack of an easily selectable phenotype for loss of the outer membrane protein. A generally usable characteristic of outer membrane proteins is their antigenicity, and their high copy number and relative ease of purification make the production of specific polyclonal or monoclonal antibodies relatively easy. However, utilizing transposon mutagenesis followed by screening of transposon-mutagenized bacteria by colony immunoblot using specific antisera (6) gives a theoretical yield of 1 knockout mutant per 4000 mutants (assuming a 1-kb gene in a 4000-kb chromosome and complete randomness of transposon delivery to the chromosome). However, it is clear that transposons do not insert into the chromosome in a completely random fashion (17), and this combined with the

requirement to screen mutants individually (due to the negative selection used) has complicated isolation of transposon-containing mutants lacking specific outer membrane proteins. For example, Tn*501* mutagenesis of the *oprP* gene was successfully accomplished using a temperature-sensitive delivery plasmid and a colony immunoblot screen (6), whereas Tn*1* and Tn*501* mutagenesis of the *oprF* gene was unsuccessful using the same procedure, even though a total of around 25,000 transposon-containing mutants were screened.

In our hands a more secure approach involves inactivation by introducing an antibiotic resistance fragment into the coding sequence of the gene on the bacterial chromosome by gene replacement after transposon mutagenesis or interposon mutagenesis of the cloned gene. Different methods have been utilized for different bacteria. However, the effectiveness of these systems is based on two characteristics of the vector that will carry the mutagenized gene: (i) it must be capable of being transferred to the recipient bacteria, and (ii) conditions must exist under which the vector cannot replicate in the recipient. Thus, selection for the antibiotic resistance marker incorporated into the gene sequence will identify those cells in which the mutated gene has recombined into the chromosome.

Transposon Mutagenesis

Two different types of vectors can be used for *E. coli* and enteric bacteria to permit transposon mutagenesis: defective transducing phages (i.e., those unable to maintain themselves in a given host) and plasmids that are temperature-sensitive for replication. A wide variety of vehicles for delivering transposons are available (6, 18), and their use in mutating an outer membrane protein gene has been described (6).

An alternative system of general relevance is Tn*phoA*, containing a Tn*5* derivative that, on transposition into a gene in the correct orientation and reading frame, results in fusion proteins comprising the N terminus of the target protein fused to the alkaline phosphatase sequence (19). The resultant fusion can give rise to increased alkaline phosphatase activity providing it is secreted (20), although other considerations are important (see Ref. 21 for discussion). Transposon mutagenesis can be effective; however, the size of transposons can be a disadvantage since it can reduce the frequency of subsequent recombinations leading to gene replacement. There are also other shortcomings associated with this approach. These include the observation mentioned above that transposons exhibit a bias for the position of integration into target DNA molecules, resulting in nonrandom insertion. In addition, transposon promoter-directed transcription can read through into adjacent DNA, thus complicating the characterization of insertion mutants (18). Also, transposons have the ability to generate DNA deletions or insertions, causing genetic instability of the mutant. Although we have had some success with transposons (6,

22), we favor an alternative approach involving *in vitro* interposon mutagenesis of the cloned gene.

Interposon Mutagenesis

Interposon mutagenesis involves utilization of a specific restriction endonuclease digestion site to open up the target gene and insert a sequence (interposon) that contains an easily selectable antibiotic resistance marker. A variety of antibiotic resistance markers have been used for *P. aeruginosa* outer membrane proteins, depending on the restriction sites available and the suitability of the antibiotic resistance gene. For example, the *oprH* gene was interrupted with a tetracycline resistance gene with flanking *Pst*I sites, since *oprH* expression in *Pseudomonas* influences susceptibility to several antibiotics (23) but not to tetracycline.

A generally applicable series of antibiotic resistance genes are provided by a series of Ω fragments (24) which range in size from 2 to 4.5 kb and contain an antibiotic resistance gene flanked by short inverted repeats carrying transcription and translation termination signals and synthetic polylinkers. The original Ω fragment containing a streptomycin/spectinomycin resistance gene was reengineered to replace this resistance gene by a series of resistance genes affecting ampicillin, kanamycin, tetracycline, chloramphenicol, or Hg^{2+} resistance. The flanking polylinkers contain *Sma*I and *Hind*III as unique restriction sites, and in some cases *Eco*RI and *Bam*HI are also unique. Thus, the antibiotic resistance genes can be inserted into plasmids linearized by restriction endonuclease digestion within the target gene. If one of the above enzyme sites is not unique, utilization of a unique endonuclease cutting site followed by filling in or removal of overhanging single-stranded DNA will permit the blunt-ended *Sma*I fragment to be inserted. Both *oprF* and *oprD* have been mutated by interposon mutagenesis (25, 26). An important advantage of interposon mutagenesis is that it is independent of the genetics of the host organism (antibiotic resistances are expressed in most species of bacteria) and it is also devoid of many of the difficulties of transposon mutagenesis.

Gene Replacement

Gene replacement requires two elements: a system that will transfer the plasmid carrying an insertion in the cloned gene and a method of forcing homologous recombination. Although introduction of plasmids by transformation or electroporation can work (27, 28), efficiencies are rarely sufficient to promote a reasonable level of gene replacement. Thus, we routinely have used plasmids carrying the origin of transfer of the IncP plasmids, and donor (mobilizing) strains carrying the transfer genes of broad host range IncP plasmid RP4 integrated in their

chromosomes. Such a system can permit almost any gram-negative bacterium to serve as a recipient for conjugative DNA transfer. Among available strains, S17-1 has been most useful because it lacks the *E. coli* K12 specific DNA restriction system, which allows the efficient transformation by the plasmid to be transferred, and it is kanamycin-sensitive, which makes selection for Tn5-containing plasmids possible (29). The vector plasmids contain the IncP-specific recognition site for mobilization and can be mobilized with high frequency from the donor strains to a broad host range of bacterial species (29).

For efficient gene replacement one requires a plasmid that has, in addition to an origin of transfer (*mob* site), an origin of replication that effectively results in plasmid replication in *E. coli*, but not in the foreign bacterium in which the gene replacement will be attempted. Thus, after transfer, the plasmid will be unable to replicate in the foreign bacterium. Under selective pressure (i.e., at a concentration of antibiotic that permits only survival of those bacteria that contain the antibiotic resistance insert within the cloned gene) only those bacteria which have undergone incorporation of the entire plasmid (by single cross-over) or recombination leading to the gene replacement (double cross-overs) will survive. These can be discriminated by the lack of incorporation of plasmid resistances in the latter and, of course, the loss of the specific outer membrane protein as revealed by SDS–PAGE and/or immunological screening. Depending on the system used, gene replacement can occur with an efficiency ranging from 5 to 50%. Plasmid systems that we have found suitable for this process are pRZ102 (30) and pNOT19 (31).

The general experimental procedure for the homologous recombination-directed insertion mutagenesis can be divided into the following steps:

1. The outer membrane protein gene of interest is cloned into the mobilizable vector before or after being mutagenized in *E. coli* by transposon insertion *in vivo* or interposon insertion *in vitro*.
2. The recombinant vector is transformed into the *E. coli* mobilizing strain S17-1 and then transferred into the original host by conjugation.
3. Transconjugants are selected using the antibiotic resistance marker inserted into the gene of interest. Counterselection against the donor can employ minimal medium since S17-1 is an auxotroph.
4. Resulting colonies are screened for the loss of the vector-encoded antibiotic resistance marker.
5. Genomic Southern hybridization analysis is performed to prove the insertion of the inserted marker at the genetic level and to confirm that only a single, mutated copy of the gene of interest is present.
6. SDS–PAGE and Western immunoblot assays of the cell envelope are performed to confirm the loss of expression of the mutagenized outer membrane protein.

Because of the generally higher level of single cross-over, Schweizer (31) developed an improved system for *P. aeruginosa* which involves using the *sacB* gene (from *Bacillus subtilis*) to encode sucrose sensitivity. Thus, after cointegrates are selected (step 3 above), cells are placed in 5% sucrose which selects positively for the excision (by single cross-over) of unwanted DNA sequences derived from the frequent whole-vector insertion events. This improves the efficiency of mutagenesis. There are two main ingredients of this system: a vector with a unique *Not*I site and a *Not*I-flanked MOB cassette which contains the origin of transfer *oriT*, the selective marker *sacB* for loss of vector sequences, and the positively selectable marker chloramphenicol resistance (since pUC-based plasmids already contain *oriT* (32), the inclusion of *oriT* on the cassette may not have been necessary).

Using this system, we inserted a kanamycin resistance Ω fragment into the *oprD* (outer membrane protein D2) gene on the *P. aeruginosa* chromosome. First, a kanamycin resistance Ω fragment is inserted into a unique *Xho*I site in the cloned *oprD* gene, then the fragment containing the *oprD*::Ω is cloned into pNOT19. Subsequently, the MOB cassette is isolated as a 5.8-kb *Not*I fragment and cloned into the unique *Not*I site on pNOT19 with *oprD*::Ω to form plasmid pND. After transformation into the *E. coli* strain S17-1 followed by conjugal transfer of pND to *P. aeruginosa*, vector integration was selected on plates containing 200 μg/ml each of kanamycin and carbenicillin. The surviving transconjugants were then grown on a plate containing 200 μg/ml of kanamycin and 5% sucrose. Since *P. aeruginosa* strains containing the *sacB* gene in single or multiple copy are highly sensitive to 5% sucrose (33), the *sacB* gene and other plasmid sequences were excised (by single cross-over) in cells surviving on this medium, leaving only the kanamycin resistance Ω insertion within the *oprD* gene. The gene replacement was confirmed by Southern analysis and examination of outer membrane proteins.

Complementation

The role of the outer membrane protein, as determined from studies of the insertion mutants created as above, should be confirmed by genetic complementation. This can prove extraordinarily difficult due to the concerns described above regarding overexpression lethality, or, in some instances, lack of expression. Indeed, the solutions to these problems usually involve the same kind of strategies discussed above for primary cloning. One caveat is that successful cloning and expression of an outer membrane protein in *E. coli* does not predict similar success in the bacterium of origin. For example, OprF is the most predominant outer membrane protein of *P. aeruginosa*, being present at around 200,000 copies per cell. Cloning the gene in *E. coli* is successful using low copy number vectors, but subcloning on medium to high copy number vectors results in DNA rearrangements (2). However, even

low copy number vectors like pRK404 and pRK415, which permitted high-level expression in *E. coli*, were not transferable to *P. aeruginosa* after cloning of the *oprF* gene. In this case, only after modification of the −10 site of the *oprF* promoter, was successful transfer to *P. aeruginosa* and expression of the *oprF* gene accomplished (4).

For the *oprH* gene, the converse, underexpression of the cloned gene in *P. aeruginosa* occurred (34). It was assumed that this reflected the loss of a regulatory mechanism in the cloned gene (possibly loss of *cis*-acting elements). In this case, enhanced expression was achieved only by including a plasmid promoter upstream of the *oprH* gene. The one regulated plasmid promoter that has been consistently useful for us in *P. aeruginosa* is the *Tol* promoter on vector pNM185 (35). However, we have also used the *lac* promoter for medium-level constitutive expression (since other elements of the *lac* regulatory system are not present in *P. aeruginosa*).

Once complementation is successfully achieved, it opens the way to structure–function studies in the native bacterium using complementation with variants isolated as described in the next section.

Construction of Outer Membrane Protein Variants

X-ray crystallography studies of outer membrane proteins have demonstrated that these proteins span the outer membrane in antiparallel β-sheet structure with long surface-exposed loops and short periplasmic loops (36, 37). Despite the tremendous amount of information that can be obtained from X-ray crystallography studies, outer membrane proteins are notoriously difficult to crystallize. Nevertheless, in the absence of a crystal structure, the membrane topology of the protein can be predicted to a certain degree of accuracy by a combination of approaches such as sequence analysis, the ability of certain regions of the protein to accommodate insertion of extra amino acids, and the analysis of mutants affecting the binding of outer membrane protein-specific monoclonal antibodies to intact cells, or the binding of bacteriophages (if the outer membrane protein studied is a phage receptor). As an example of these approaches, the prediction of the location of surface-accessible loops of the *P. aeruginosa* outer membrane protein OprF is discussed below using a combination of genetic and immunological approaches.

Linker Insertion Mutagenesis

The procedure that we have employed to study the membrane topology of OprF results in the insertion of a four amino acid linker into various regions of the protein. The basic strategy involves the use of a kanamycin resistance cartridge flanked by symmetric restriction enzyme sites contained in the pUC4K series of plasmids (38).

Initially, the plasmid carrying the cloned *oprF* gene is linearized separately by partial digestion with various blunt-end cutting restriction enzymes utilizing conditions that result in an average single cut per plasmid copy, followed by the ligation and recircularization with a blunt-ended fragment from pUC4K containing the kanamycin resistance cartridge (39). After transformation, recombinants are selected on media containing kanamycin and screened with an OprF C terminus-specific monoclonal antibody on a colony immunoblot to select for insertion within the *oprF* coding sequence. Plasmid DNA from the kanamycin-resistant clones that do not react with this specific monoclonal antibody are extracted and digested with *Pst*I, which recognizes sites in the flanking sequences of the kanamycin resistance cartridge. The religated plasmids from clones that recover immunological reactivity with OprF-specific antibodies and are kanamycin-sensitive carry a 12-base pair insert at the sites originally interrupted by the kanamycin resistance cartridge, and hence result in an in-frame insertion of four amino acids in the translated protein. Therefore, depending on the number of cleavage sites recognized by the restriction enzymes used to linearize the *oprF*-containing plasmid, a series of linker insertion mutants can be generated, each having the 12-base pair insert at a different location within the *oprF* gene. This method allows one to identify sites in the protein that permit the insertion of extra amino acid residues. These so-called "permissive sites" are hypothesized to be located in the surface-exposed loops of the proteins due to a lesser degree of spatial constraints in those regions. Consistent with this, all known insertion sites in the PhoE protein occur within the surface-exposed loop regions indicated by the crystal structure (37). The permissiveness of the linker insertion sites in the linker variants can be evaluated by their cellular locations (assessed by cell fractionation), mobility on SDS–PAGE, reactivity with specific monoclonal antibodies, and proper membrane configuration (as indicated by sensitivity to proteases) and by the surface exposure of certain regions (by immunofluorescence labeling of intact cells with specific antibodies). After all these criteria have been investigated, permissive sites for the insertion of the extra amino acids are defined and the *Pst*I site carried in the 12-base pair insert can be used for the insertion of a defined foreign epitope as discussed below. Other unique sites flanking the kanamycin resistance cartridge are available for insertion mutagenesis by using other members of the pUC4K series of plasmids (38).

Epitope Insertion

The linker mutagenesis with the kanamycin resistance cartridge can also create a unique *Pst*I site (provided *Pst*I sites were deleted from the original plasmid) which is available for the insertion of a foreign epitope. The choice of the foreign epitope will depend on the availability of the amino acid sequence of the epitope as well as the availability of detecting reagents such as epitope-specific monoclonal antibod-

ies. Hofnung (39) has discussed a wide range of suitable epitopes. Having decided on an epitope, oligonucleotides corresponding to the amino acid sequence of the epitope can be synthesized and inserted into the unique restriction enzyme sites. Recombinants expressing the hybrid proteins can be selected by colony immunoblot for their reactivities with both outer membrane protein-specific and epitope-specific monoclonal antibodies. The surface location of the insertion site can then be further confirmed by the surface exposure of the inserted epitope, which can be determined by immunofluorescence labeling of intact cells expressing the hybrid protein with antibodies that are specific for the epitope. Moreover, if the inserted epitope contains a trypsin-sensitive site, an increase in trypsin cleavage targets of the hybrid protein in intact cells can also be used as an indication of surface location of the insertion regions (40).

Deletion Construction

The mutants generated by the linker insertion mutagenesis mentioned above can also be used for the construction of deletion mutants by inserting an oligonucleotide adaptor carrying stop condons in all three reading frames into the *Pst*I sites of the linker insertion mutants. Depending on the positions of the *Pst*I sites in the mutants, C-terminal truncated proteins of different lengths will be translated. Mutants with internal deletions of different sizes can also be generated by ligating the N terminus and C terminus of different linker mutants at their unique *Pst*I sites. The loss of reactivity of the internal deletion mutants with any surface-specific antibodies will suggest the surface localization of the deleted sequences.

Alternative routes to deletion construction involved direct excision of restriction fragments (although convenient sites are often rare) and Tn*phoA* mutagenesis. In the latter case it was found that when the transposon Tn*phoA* was inserted out-of-frame or in the inverse orientation, a defined series of carboxy-terminal deletions were created with 1–20 extra amino acids at the C terminus (depending on the reading frame and orientation of Tn*phoA*) due to read-through to a stop codon in the transposon (19). However, in some outer membrane proteins, especially the nonspecific porins like PhoE, the C terminus is critical for stability of the protein, and deletion mutagenesis would not be expected to work (41).

Functional and Antigenic Properties of Mutated Clones

Since outer membrane proteins serve a variety of functions, functional studies of the mutants will provide information on how different segments of the protein are involved in particular functions. For example, deletion studies of outer membrane proteins such as OmpA and PhoE have demonstrated that certain stretches of amino

acids are required for efficient export or translocation across the outer membrane (42, 43). In addition, insertion studies of PhoE have provided evidence that the third loop of the protein is located within the pore channel, which is consistent with the data generated from X-ray diffraction analysis of the porin crystal (44). For epitope insertion mutants, studies of the immunogenicity of the inserted epitope at different regions of the protein will give insight into the effect of flanking amino acids on the presentation of an epitope to the immune system and should add to our understanding of the mechanism of antigen presentation in general.

References

1. J. Smit and H. Nikaido, *J. Bacteriol.* **135,** 687 (1978).
2. W. Woodruff, T. R. Parr, R. E. W. Hancock, L. F. Hanne, T. I. Nicas, and B. J. Iglewski, *J. Bacteriol.* **167,** 473 (1986).
3. R. Munson and R. W. Tolan, *Infect. Immun.* **57,** 88 (1989).
4. R. S. Y. Wong, H. Jost, and R. E. W. Hancock, *Mol. Microbiol.*, in press (1993).
5. P. van der Ley, H. Amensz, J. Tomassen, and B. Lugtenberg, *Eur. J. Biochem.* **147,** 401 (1985).
6. K. Poole and R. E. W. Hancock, *Mol. Gen. Genet.* **202,** 403 (1986).
7. H. Huang, R. J. Siehnel, F. Bellido, E. Rawling, and R. E. W. Hancock, *FEMS Microbiol. Lett.* **76,** 267 (1992).
8. A. Bell and R. E. W. Hancock, *J. Bacteriol.* **171,** 3211 (1989).
9. P. Matsudaira, *J. Biol. Chem.* **262,** 10035 (1987).
10. M. Tusuda, S. Harayama, and T. Iino, *Mol. Gen. Genet.* **196,** 494 (1984).
11. R. J. Siehnel, E. A. Worobec, and R. E. W. Hancock, *J. Bacteriol.* **170,** 2312 (1988).
12. T. K. Misra, M. L. Brown, D. C. Fritzinger, R. D. Pridmore, W. M. Barnes, L. Haberstroh, and S. Silver, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 5975 (1984).
13. R. J. Siehnel, E. A. Worobec, and R. E. W. Hancock, *Mol. Microbiol.* **3,** 347 (1988).
14. A. Filloux, M. Bally, C. Soscia, M. Murgier, and A. Lazdunski, *Mol. Gen. Genet.* **212,** 510 (1988).
15. A. Bell, M. Bains, and R. E. W. Hancock, *J. Bacteriol.* **173,** 6657 (1991).
16. C. D. O'Conner and K. N. Timmis, *J. Bacteriol.* **169,** 4457 (1987).
17. P. Prentki and H. M. Krisch, *Gene* **28,** 393 (1984).
18. N. Kleckner, J. Roth, and D. Botstein, *J. Mol. Biol.* **116,** 125 (1977).
19. R. L. Finnen, N. L. Martin, R. J. Siehnel, W. A. Woodruff, M. Rosok, and R. E. W. Hancock, *J. Bacteriol.* **174,** 4977 (1992).
20. C. Manoil and J. Beckwith, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 8129 (1985).
21. C. K. Murphy and P. E. Klebba, *J. Bacteriol.* **171,** 5894 (1989).
22. E. A. Worobec, R. J. Siehnel, P. Gladman, and R. E. W. Hancock, *FEMS Microbiol. Lett.* **52,** 235 (1988).
23. M. L. Young, M. Bains, A. Bell, and R. E. W. Hancock, *Antimicrob. Agents Chemother.* **36,** 2566 (1992).
24. R. Fellay, J. Frey, and H. Krisch, *Gene* **52,** 147 (1987).

25. W. A. Woodruff and R. E. W. Hancock, *J. Bacteriol.* **170,** 2592 (1988).
26. H. Huang and R. E. W. Hancock, *J. Bacteriol.* **175,** in press (1993).
27. D. Berry and A. M. Kropinski, *Can. J. Microbiol.* **32,** 436 (1986).
28. M. A. Farinha and A. M. Kropinski, *FEMS Microbiol. Lett.* **70,** 221 (1990).
29. R. Simon, U. Priefer, and A. Pühler, *Bio Technology* **1,** 784 (1983).
30. R. A. Jorgensen, S. J. Rothstein, and W. S. Reznikoff, *Mol. Gen. Genet.* **177,** 65 (1979).
31. H. P. Schweizer, *Mol. Microbiol.* **6,** 1195 (1992).
32. P. H. Pouwels, B. E. Enger-Valk, and W. J. Brammer, "Cloning Vectors: A Laboratory Manual." Elsevier, Amsterdam, 1985.
33. J. L. Reid and A. Collmer, *Gene* **57,** 239 (1987).
34. A. Bell, M. Bains, and R. E. W. Hancock, *J. Bacteriol.* **173,** 6657 (1991).
35. N. Mermod, J. L. Ramos, P. R. Lehrbach, and K. N. Timmis, *J. Bacteriol.* **167,** 447 (1986).
36. M. S. Weiss, U. Abele, J. Weckesser, W. Welte, E. Schiltz, and G. E. Schulz, *Science* **254,** 1627 (1991).
37. S. W. Cowan, T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R. A. Paupit, J. N. Jansonius, and J. P. Rosenbusch, *Nature (London)* **358,** 727 (1992).
38. J. Vieira and J. Messing, *Gene* **19,** 259 (1982).
39. D. O'Callaghan, A. Churbit, P. Martineau, C. Leclerc, S. van der Werf, C. Nauciel, and M. Hofnung, *Res. Microbiol.* **141,** 963 (1990).
40. J. Ronco, A Charbit, and M. Hofnung, *Biochemie* **72,** 183 (1990).
41. D. Bosch, M. Scholten, C. Verhagen, and J. Tommassen, *Mol. Gen. Genet.* **216,** 144 (1989).
42. R. Fruedl, M. Klose, and U. Henning, *J. Bioenergetics Biomembr.* **22,** 441 (1990).
43. D. Bosch, W. Voorhout, and J. Tommassen, *J. Biol. Chem.* **263,** 9952 (1988).
44. M. Struyve, J. Visser, H. Adriaanse, R. Benz, and J. Tommassen, *Mol. Microbiol.* **7,** 131 (1993).