Development of a cloning system for the expression of mycoplasma genes in *Escherichia coli*

by

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DEDICATION

This manuscript is dedicated to my husband Mark Seth Smiley whose love and support have made my work possible and to Jesus Christ my Savior who has blessed me with the abilities and the strength to work for His glory.

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GENERAL INTRODUCTION

An Explanation of the Dissertation Organization

This thesis includes two separate manuscripts which have been submitted for publication. The manuscripts are preceded by a literature review and are followed by a general summary. The references cited in the literature review and the general summary follow the general summary.

Literature Review

Mycoplasma gene expression libraries

The mycoplasmas (Class *Mollicutes*) lack a cell wall and are the smallest prokaryotic organisms. They have limited biosynthetic capabilities, are ubiquitous parasites of mucous membranes and joints, and are frequently implicated in respiratory and urogenital tract infections in a variety of mammalian and avian species (73). Genetic analysis of these organisms has been limited due to their poor growth characteristics, their dependence on expensive undefined media, their inefficient transformation with DNA, and a lack of suitable cloning vectors. Protocols for the cloning and expression of mycoplasma genes in *Escherichia coli* have been developed in an attempt to circumvent the obtacles which interfere with the direct genetic manipulation of mycoplasmas. Libraries of mycoplasma chromosomal DNA have been constructed using Lambda Charon 4A (90), Lambda Charon 28 (78), Lambda gt11 (1, 39,15, 56), Lambda Zap (81, 88), Lambda EMBL3 (43, 91), and plasmid vectors such as pBR322 (78), pBR328 (54), pEx1,-2, and -3 (25), pHC79 (58), pEx-3x, pEx34 (50), pEx29 (42), as well as cosmid vectors such as pCos (83, 94). Immunological screening of libraries has been used successfully to identify genes that encode antigenic mycoplasma proteins which are expressed in *E. coli*. (1, 12, 15, 37, 39, 45, 48, 52, 56, 81, 84, 85).

The molecular and genetic techniques which have been developed for use with *E. coli* cloning hosts have great potential for the examination of mycoplasma genes. The small genome size of mycoplasma species (26) facilitates the screening of chromosomal libraries because fewer clones are needed to generate a representative library. Monoclonal and polyclonal antibodies can be used in library screenings to identifiy and characterize surface components that may mediate attachment to host cells, modulate the immune response, or play some other role in colonization or virulence. Host-parasite interactions and disease pathogenesis can be examined at the level of surface protein structure, as well as at the level of virulence gene organization and regulation. Cloned DNA fragments can be used to localize genes on the chromosome and to investigate the phylogenetic relationships among species on the basis of genomic organization. Mycoplasmal protein expression in *E. coli* can also facilitate the identification and large scale production of vaccine antigens for further study or commercial applications.

UGA codon usage in mollicutes

Mycoplasma gene expression in *E. coli* using traditional cloning techniques has been hindered by the unusual codon usage pattern displayed by many mycoplasma species. Most mycoplasmas utilize the UGA termination codon to encode the amino acid tryptophan. Deviation from the typical prokaryotic genetic code was first observed in *M. capricolum* by Yamao et al. (97). *M. capricolum* contains TGA triplets in many sites that correspond to tryptophan TGG triplets in homologous *E. coli* genes. The TGG triplet is also found in *M. capricolum* protein coding sequences, but at a lower frequency than TGA (64, 97). Translation of synthetic messenger RNA, containing in-frame UGA codons, in cell extracts from *M. capricolum* has demontrated that UGA is read as a tryptophan in this organism (61). Two $tRNA^{Trp}$ with anticodons UCA and CCA are encoded by a single operon in the *M. capricolum* genome. The UCA anticodon decodes both UGA and UGG codons, while the CCA anticodon

reads only UGG (2, 96, 97). These tRNA species are maintained at different levels within the cell, and they appear to differ in their ability to accept tryptophan (96).

The *M. capricolum* tRNA genes were used as probes in the cloning and sequence determination of the tRNA coding regions from *M. pneumoniae*, *M. genitalium*, and *M. gallisepticum* (33). *M. gallisepticum*, like *M. capricolum*, was found to contain two $tRNA^{trp}$ with anticodons UCA and CCA. *M. pneumoniae* and *M. genitalium* each was found to possess a single $tRNA^{trp}$ with the UCA anticodon. The nucleotide sequences of the *M. pneumoniae* P1 adhesion, the *M. genitalium* MgPa attachment protein, and the *tuf* gene of *M. gallisepticum* further suggest that UGA encodes tryptophan in these organisms. Synthetic oligonucleotides specific for each $tRNA^{trp}$ gene of *M. capricolum* have been used to probe the genome of *Spiroplasma citri* and have demonstrated the existence of a gene homologous to the gene encoding a tRNA with the UCA anticodon, but not one with the CCA anticodon (13). In addition, analysis of the coding sequences from the spiroplasma SpV4 virus revealed that even the largest open reading frame was not extensive enough to encode the capsid protein unless UGA was recognized as a sense codon (75).

A cryptic plasmid of *M. mycoides* was found to contain two TGA triplets in positions that were occupied by TGG in a homologous staphylococcal plasmid (6). A TGA triplet was also discovered in a cloned gene in a position corresponding to tryptophan in a putative transport protein of *M. hyorhinis* (16). Three TGA triplets were correlated with tryptophan residues in the *M. arginini* arginine deiminase gene (43), and the same gene in *M. hominis* PG21 was found to encode five UGA codons (24). A comparison of the sequence of the urease gene of *Ureaplasma urealyticum* with that of jack bean urease implicated UGA as a tryptophan codon in this organism as well (7).

Although the use of UGA as a sense codon appears to be quite common in the class *Mollicutes*, the extent of its usage has not been determined. One exception is found in the non-sterol requiring genus *Acholeplasma*. A single $tRNA^{trp}$, which contains the anticodon CCA and

which reads the UGG codon, has been found in *A. laidlawii* (89). The code change of UGA for tryptophan probably occured in the *Mycoplasma/Spiroplasma* lineage after the separation of these bacteria from *Acholeplasma* (89).

Development of alternative codon usage

A definitive explanation for the existance of altered codon usage in mycoplasma species has not been established, although several hypotheses including mutation pressure and/or selection, have been proposed. Except for *M. pneumoniae*, mycoplasmas possess an extremely AT-rich genome with a G+C content of 24-35 mol% (74). Differences in GC/AT content have generally been attributed to genes that are responsible for DNA replication and repair (57). In *E. coli*, several genes are known to generate G:C to A:T transversions (59), and it has been suggested that variations in DNA repair systems might also be responsible for biased mutation pressure in the class *Mollicutes* (36, 95). *M. capricolum*, with an AT content of 75%, displays a codon bias; A- or U- containing codons are preferentially used, and 91% of the codons have A or U at the third position (64). Analyses of the codon usage in the spiralin gene of *S. citri* (12), the urease gene of *U. urealyticum* (7), and the arginine deiminase gene of *M. hominis* PG21 (24) revealed a preferential use of A- or U- rich codons in these organisms as well. It has been suggested that this codon bias may be the result of an AT-biased mutation pressure which replaces GC pairs with AT pairs. Preferential use of certain codons would accomodate an AT-rich genome without altering the protein composition of the organism (57).

A model has been proposed whereby a series of nondeleterious changes resulting from AT-mutational pressure could have enabled the establishment of UGA as a tryptophan codon in the mycoplasmas (37, 65, 66, 67, 97). AT-biased mutations could have converted UGA termination codons to UAA. At this stage, the release factor which interacted with UGA and UAA must have been deleted or become specific for UAA so that UGA could become unassigned. A copy of $tRNA_{CCA}$ may been have mutated to $tRNA_{UCA}$ after duplication of

the original gene. The existance of $tRNA_{UCA}$ would have allowed the conversion of the UGG codon to the UGA codon under AT pressure. The report by Yamao et al. that *M. capricolum* contains two highly similar tRNAs with anticodons UCA and CCA in a tandem arrangement is consistant with this model (96). Since the UCA anticodon is capable of reading the UGG codon, the CCA anticodon is no longer needed, and it is not found in some mycoplasma species such as *M. pneumoniae* and *M. genitalium* (33).

Analysis of the codon usage of the P1 protein operon from *M. pneumoniae* demonstrated that preferential usage of A- or U- rich codons does not occur in this operon (30). Hu et al. suggested that the replacement of $tRNA_{CCA}$ with $tRNA_{UCA}$ in *M. pneumoniae* is probably due to a simplified decoding system similar to the one seen in mitochondria. Mitochondrial development appears to have been influenced by a strong pressure to reduce genome size (3, 23). This decrease has been correlated with a reduction in the number of tRNA genes. An unconventional reading hypothesis has been proposed by Lagerkvist to explain how fewer tRNAs can decode all of the codons in the genetic code (47). The sequences of the tRNA genes in *M. mycoides* suggest a similar lack of discrimination in reading certain codon families, although information on codon usage in this species is not available (78). Mycoplasmas exhibit other similarities to mitochondria in that they appear to be related to Gram positive bacteria, they can multiply as intracellular parasites, and they have reduced genomes. This has led Anderson and Kurland to speculate that both mitochondria and mycoplasmas have evolved under pressure to reduce genome size, with a corresponding simplification of tRNA complexity and codon reassignment, as an adaptation to their intracellular existence. They have proposed that codon reassignments are probably the result of a combination of biased mutation pressure and selective forces (3). The observation that the translation system of bacteria is optimized under favorable growth conditions through a reduction in the complexity of the tRNA population suggests that codon bias may provide a selective advantage (17).

Use of *E. coli* as a cloning host for mycoplasma sequences

The UGA codons in transcripts of mycoplasma sequences are recognized as termination signals in *E. coli*, resulting in premature truncation of cloned proteins. Many of these truncated proteins escape immunological detection in library screenings. Initial attempts to clone and express the *M. pneumoniane* P1 adhesion in *E. coli* indicated that the protein was not completely expressed (15, 32, 79, 88). After the gene was cloned by a method that did not require protein expression, sequencing of the P1 gene revealed the presence of 21 TGA triplets (88). Similar efforts to express the *M. pneumoniae* cytadherence accessory protein HMW3 resulted in a truncated product due to the presence of 2 TGA triplets within the coding sequence (62, 63). Efforts to obtain expression in *E. coli* of the urease genes from *U. urealyticum* were also unsuccessful (8), and two of the three open reading frames in the cloned sequence were subsequently found to contain 8 TGA triplets (7).

A variety of approaches have been used to surmount the difficulties inherent in cloning mycoplasma gene sequences due to inadequate protein expression. Immunological screening of expression libraries has been used to isolate clones containing epitopes from larger proteins. Positive clones have then been used as probes in DNA hybridization studies to isolate the complete genes without requiring protein expression (32). Expression vectors which generate fusions with β-galactosidase (44), bacteriophage MS2 RNA replicase (42, 85), or mouse dihydrofolate reductase (70), have provided recombinant proteins with improved stabilities and detectable molecular weights on immunoblots, even when only a small portion of the protein was expressed. Genes have also been identified by hybidization with DNA probes whose sequences were derived from cloned homologous genes (7, 8) or were based upon the sequence of purified proteins (43, 88). Tn*phoA* fusions to cloned sequences have been used to identify membrane-associated protein genes, since only the infrequent occurance of a TGA triplet in a signal sequence limits detection (98). Expression of mycoplasma proteins has been obtained in some instances after cloning and sequencing, by site-directed mutagenesis of TGA

triplets (31, 60). Although these approaches have enabled the isolation of certain mycoplasma genes, premature truncation may prevent gene identification in cases where expression is essential for detection because partial protein products do not contain a crucial epitope or lack functional activity.

Use of opal suppressor tRNAs in the readthrough of UGA codons

An *E. coli* cloning host that could ignore the termination signals within mycoplasma coding sequences would simplify the gene isolation process and could allow the detection of genes that might never be observed with other methods. Due to the poor growth characteristics and expensive media requirements of mycoplasma species, protein expression in *E. coli* would also be advantageous for the large scale expression of mycoplasma antigens for scientific study or vaccine preparation. Opal suppressor alleles encoding tRNAs that recognize the UGA codon have been incorporated into *E. coli* cloning hosts for the expression of mycoplasma sequences. Renbaum et al. (76) demonstrated the expression in *E. coli* of a spiroplasma gene encoding a CpG DNA methylase by using the opal suppressor allele *trpT*176. Upon sequence analysis of the cloned gene, four TGA triplets were detected. Proft and Herremann have used suppressors to overcome alternate codon usage in cloned sequences from *M. pneumoniae* (64).

We have developed an expression system that also uses *trpT*176 as a source of opal suppressor activity. Raftery et al. generated the *trpT*176 allele by inducing an anticodon mutation in the cloned *trpT* gene which encodes the wild type $tRNA^{trp}$ (71). The *trpT*176 allele encodes a tRNA that also appears to be charged with tryptophan, but whose altered anticodon recognizes the UGA termination codon. This allele was demontrated to be a relatively strong suppressor of UGA nonesense mutations in a *lacI-lacZ* fusion, although the context of the mutation influenced the efficiency of suppression. We have established regulation of the *trpT*176 allele by subcloning it behind the strong IPTG-inducible *trc* promoter from the prokaryotic expression vector pTrc99 (Pharmacia). The $lacI^q$ allele was included on the same

episome to generate sufficient repressor such that basal expression of the suppressor activity is minimized during library construction, but can be induced at the time of cloned gene expression. By utilizing cloning vectors that are also regulated by *lacI*, suppressor activity and potentially detrimental cloned gene expression can be induced simultaneously.

Regulation of supression is desirable since readthrough of the termination signals in host transcripts might be predicted to be detrimental to cell survival due to a lack of functional activity in the abnormally extended products. The prevalence of UGA termination codons within *E. coli* transcripts has been investigated by tabulating their occurance within reported gene sequences. An analysis of 165 *E. coli* genes indicated that genes with high sense-codon bias usually ended in UAA. Since sense codon usage varies between genes according to the level of gene expression, there existed a strong bias in favor of UAA in highly expressed genes. UGA was used with an increasing frequency in genes with a lower sense-codon bias, and UAG was always used infrequently (81). An analysis of codon usage in 1187 *E. coli* genes which were listed in GenBank revealed that UAA accounted for 67% of the termination codons in these sequences, whereas UGA and UAG represented 27% and 6.7%, respectively (92). Another examination of the stop codons and surrounding sequences in 862 *E. coli* genes again revealed a strong bias in the stop codon usage; 92% of the highly expressed genes terminated in UAA, while only 38% of the poorly expressed genes were terminated by UAA. Over one quarter of the genes in the database terminated in UGA (9).

The severity of the damage inflicted on an *E. coli* cell by UGA readthrough has not been ascertained. Some suppressor strains can grow without apparent consequences, and Rich et al. found that strains carrying amber and ochre suppressor tRNAs did not have any detectable changes in the molecular weight distribution of proteins compared to wild type *E. coli* (49). Rich predicted that the host might be protected from the effects of suppressor alleles by the occurance of more than one termination codon in tandem. Indeed, seventy-five percent of the genes in the data base of 862 genes were found to terminate in a double stop (9). In the

instances where single stop codons indicate termination, Rich suggested that the surrounding sequences in the bacterial chromosome might provide an additional signal that favors termination over suppression (49).

UGA suppression efficiency

Despite the availability and use of opal suppresor tRNAs to enhance the expression of mycoplasma genes, suppressors generally function with a relatively low efficiency that varies depending upon the circumstances. The effectiveness of suppressor readthrough versus termination of protein synthesis depends upon a number of poorly defined factors such as the nature of the suppressor tRNA, messenger RNA context effects, interaction with the other tRNAs on the ribosome, and competition with the release factor (9). The efficiency of the *trpT*176 allelle has been demonstrated to be context dependent, with the efficiency at one site being reduced by two-thirds compared to another (71). The introduction of base changes in the codons adjacent to a UGA codon have demonstrated that third position base changes in codons 5' and 3' to UGA codons do affect UGA suppression and that an A residue immediately downstream favors suppression (10, 53). It is uncertain how context influences nonsense suppression; it could affect the efficiency of the tRNA suppression or the termination event or both. The adjacent mRNA sequences might alter mRNA structure (9), influence the interaction of the tRNAs on the ribosome (72, 84), or stabilize the codon/anticodon interaction through base stacking (86). Context effects might depend upon interaction between the stop codons and 16s rRNA (56). There is also evidence to suggest that context influences release factor activity in that the relative affinities for release factor-1 (RF-1) and release factor-2 (RF-2) at UAA codons are subject to context (51).

Enhancement of *trpT*176 suppression efficiency

We have investigated the feasiblity of using cloned opal suppressor tRNAs in conjunction with other mutations that also exhibit readthrough of UGA codons in an attempt to enhance suppressor efficiency. The *prfB* gene encodes the peptide chain release factor 2 (RF-2) in *E. coli*. (11, 93). RF-2 is a protein which binds to the ribosome, recognizes UGA and UAA termination codons, and facilitates termination of protein synthesis by triggering the release of the nascent polypeptide. Another release factor, RF-1, catalyzes termination at UAA and UAG codons (14, 80). Approximately 60 percent of the total termination activity at UAA is due to RF-1, whereas RF-2 accounts for 40 percent. RF-2 is more active at UGA than at UAA (80).

The *prfB3* mutation was generated by transduction of *E. coli* with hydroxylamine-mutagenized P1 phage with selection for a closely linked Tn*10* transposon. The mutation was isolated on the basis of its ability to suppress a *lacZ(UGA)* mutation (38). Sequencing of the mutant allele revealed a guanine to adenine change at position 428, substituting asparagine for aspartate 143 (52). It is not known how the *prfB3* mutation causes UGA supression. Studies of the basal level regulation of the *trp* operon (77) and the *pheA* operon (21) demonstrated that the *prfB3* mutation increased transcriptional termination 2-fold at the *trp* and the *pheA* attenuators. It was postulated that a reduced level of RF-2 activity led to ribosomal stalling at UGA stop codons in the leader RNA, generating an increased formation of a transcriptional termination structure. Reducing the cellular concentration of RF- 2 also generates UGA suppressor activity (39), and it has been assumed that any reduction in the activity of RF-2 slows down the termination process and leads to abnormal pausing of the ribosome which may cause misreading of UGA codons and other translational errors (52).

Release factors compete with suppressor tRNAs for recognition of termination codons (5, 20, 51). A plasmid carrying the cloned *prfB* gene reduced UGA suppression by *trpT*176 in vivo (93). We propose that a mutation such as *prfB3* which hinders RF-2 termination activity may provide the suppressor tRNA with a competitive advantage and may therefore augment the

insertion of tryptophan at UGA sites. Use of a strain with a temperature sensitive RF-1 has been shown to significantly improve the activity of relatively weak amber suppressor tRNAs (41).

The sequence of events which occurs during termination of protein synthesis has not been precisely defined. Release factors are known to play an important role, but the involvement of ribosomal RNA has also been proposed (56, 69, 82). In addition to altered tRNA alleles and release factor mutations, certain lesions in ribosomal RNA also facilitate translational readthough of UGA codons. We have investigated the potential usefulness of such a mutation for mycoplasma gene expression. Murgola et al. isolated a spontaneous mutation in a chromosomal gene that functioned as a suppressor of a *trpA*(UGA211) nonsense mutation (56). Subsequent cloning and sequencing determined that the mutation consisted of the deletion of base C1054 in the 16S rRNA. This mutation was determined to be necessary and sufficient for UGA suppression. Additional studies revealed that this lesion causes the ribosome to act as a "super " suppressor by enhancing the activites of tRNA suppressors at all three nonsense codons. Suppression requires the presence of a tRNA to read the nonsense codon. UGA is the only termination codon for which a weak suppression by $tRNA^{trp}_{UGG}$ occurs naturally; readthrough at the other two termination codons requires the addition of suppressor tRNAs (68). We introduced the *trpT*176 allele into a strain posssessing this "super suppressor" phenotype, anticipating an increase in efficiency of UGA readthrough.

Analysis of system efficacy

UGA suppressor strains were employed in the expression of portions of the P1 structural gene from *M. pneumoniae*. *M. pneumoniae*, the etiological agent of primary atypical pneumoniae in children and young adults, colonizes the mucosal surface of the respiratory tract (34). The P1 protein is a hydrophobic integral membrane protein which clusters at the tip of an attachment organelle and mediates cell adherence to the ciliated respiratory epithelium (4, 18,

28, 29, 48). Mutants which lack P1 or other proteins involved in its localization or anchoring are avirulent (4, 45, 46).

The P1 adhesion is an appropriate trial antigen to explore the feasibility of our expression system because it typifies proteins that might be discovered using this approach. The P1 protein is a critical virulence determinant, as well as a major immunogen, which stimulates antibody production in experimental animals and human patients (27, 28, 48). The P1 gene, which has been cloned and sequenced (32, 88), encodes 21 UGA codons. Early attempts to express the P1 protein in *E. coli* met with limited success (15, 32, 79, 88), and cloning was accomplished by using an approach that did not require complete protein expression (32, 88). Based upon sequence information, we subcloned a fragment containing four dispersed TGA triplets which encoded a 72 kDa protein. Hydrophilicity plots of the protein sequence had predicted antigenic sites in this region which might be used to detect protein products with antisera produced against the P1 protein (88). Epitope mapping of overlapping peptides using the sera of infected patients had also identified some immunodominant antigenic domains within the cloned fragment (35).

After demonstrating readthrough of four UGA codons using a subcloned fragment of the P1 gene, we also attempted expression of the entire P1 gene, with or without the presence of its signal sequence. The first 59 amino acids of the P1 protein appear to compose a leader peptide consisting of positively charged amino acids followed by a hydrophobic core and then a hydrophilic core (32, 88). Based upon the derived amino acid sequence of the P1 protein, the mature protein has a calculated molecular weight of 169,758 daltons after the removal of the signal peptide. A possible cleavage site that resembles the most common consensus sequence is found at amino acid twenty instead of at the traditional location at the end of the signal peptide. Since the steps involved in processing the P1 protein have not been elucidated, we were uncertain whether the signal peptide would be cleaved in an *E. coli* host. Inefficient processing might lead to the generation of several intermediate forms with different molecular weights, as

was seen with spiralin from *S. citri* (54, 55). If the P1 protein translocation signals were recognized in *E. coli*, insertion into the membrane might be lethal to the cell. We therefore included in our analysis a cloned P1 gene that did not contain the signal sequence.

Our expression system was designed for the screening of mycoplasma genomic libraries. We assessed the effectiveness of the system by screening lambda libraries containing gene sequences from *M. hyopneumoniae*. *M. hyopneumoniae* is the causative agent of the economically important swine disease, mycoplasmal pneumonia. *E. coli* cloning hosts have been used to express *M. hyopneumoniae* sequences in an attempt to isolate epitopes with diagnostic or vaccine potential (1, 40, 42, 87). However, there have been suggestions that *M. hyopneumoniae* utilizes the unusual codon usage pattern which is characteristic of most other mycoplasma species (19, 42). Premature truncation in *E. coli* may preclude the isolation of valuable genes by concealing critical epitopes or functional domains. We screened Lambda Fix and Lambda Zap II libraries, containing *M. hyopneumoniae* DNA, on suppressor strains and compared the results to those obtained on nonsuppressor strains.

PAPER 1. ENHANCED READTHROUGH OF OPAL (UGA) CODONS AND EXPRESSION OF *MYCOPLASMA PNEUMONIAE* P1 EPITOPES IN *ESCHERICHIA COLI*

Enhanced readthrough of opal (UGA) codons and expression of *Mycoplasma pneumoniae* P1 epitopes in *Escherichia coli*

(stop codon, suppressor tRNA, *trpT*176)

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Abbreviations: Ap, ampicillin; bp; base pair(s); CAT, chloramphenicol acetyltransferase; dNTP, deoxyribonucleoside triphosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; LB, Luria-Bertani (medium); *M., Mycoplasma*; oligo, oligodeoxyribonucleotide; nt, nucleotide(s); PBS, phosphate-buffered saline (0.01 M sodium phosphate 0.14 M NaCl pH7.3); PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; RF-2, release factor 2; RBS, ribosome-binding site; SDS, sodium dodecyl sulfate; TAE, tris-sodium acetate-ethylene diamine tetraacetic acid (buffer); Tc, tetracycline; TS, tris-saline buffer (0.01 M Tris 0.14 M NaCl pH 7.4); TSN, TS - Tween 20 buffer (TS 0.05% Tween 20); XGal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside.

SUMMARY

Expression of mycoplasma sequences in *Escherichia coli* is often hindered by an unusual mycoplasmal codon usage pattern; the UGA termination codon is utilized for tryptophan. This may result in premature truncation of cloned proteins and may prevent the detection of many cloned genes. To circumvent this translation barrier, we have developed an expression system for the production of mycoplasma proteins in *E. coli*. The efficiency of an opal suppressor tRNA (*trpT*176) was augmented with other suppressor mutations [*prfB*3 or *rrsB*(SuUGA-ΔC1054)] which influence termination events. System efficacy was analyzed by employing suppressor mutations in the expression of TGA-containing sequences from the P1 protein gene of *M. pneumoniae*.

INTRODUCTION

The mycoplasmas (Class *Mollicutes*) lack a cell wall and are the smallest prokaryotic organisms. They have limited biosynthetic capabilities, are ubiquitous parasites of mucous membranes and joints, and are frequently implicated in respiratory and urogenital tract infections in a variety of mammalian and avian species (Razin, 1978). Genetic analysis of these organisms has been limited due to poor growth characteristics, dependence on expensive undefined media, inefficient transformation with DNA, and a lack of suitable cloning vectors. Mycoplasma gene expression in *Escherichia coli* using traditional cloning techniques has been hindered by the unusual codon usage pattern displayed by many mycoplasma species. Most mycoplasmas utilize the codon UGA to encode the amino acid tryptophan (Bergemann et al., 1989; Dudler et al., 1988; Inamine et al., 1990; Kondo et al., 1990; Renaudin et al., 1987; Yamao et al., 1985). In *E. coli*, this codon is recognized as a termination signal resulting in premature truncation of cloned mycoplasma proteins. Many of these truncated proteins escape immunological detection in library screenings (Blanchard, 1990; Blanchard and Barile, 1989; Dallo et al., 1988; Inamine et al., 1988; Ogle et al., 1992; Su et al., 1987). In this paper, we describe the development of an expression system which overcomes this translational barrier by facilitating readthrough of UGA codons in *E. coli*.

Renbaum et al. (1990) demonstrated expression in *E. coli* of a spiroplasma CpG DNA methylase gene encoding four UGA codons by using the opal suppressor allele *trpT*176. The tRNA produced from this allele is charged with tryptophan, but its anticodon recognizes the UGA termination codon (Raftery et al., 1984). In our system, we have minimized basal transcription of *trpT*176 by the inclusion of *lacI*^{q} on the same episome. By utilizing cloning vectors that are also regulated by *lacI*, suppressor activity and potentially detrimental cloned

gene expression can be coordinately regulated. A p15A origin of replication was used in the construction to provide compatibility with pMB1-derived cloning vectors.

Despite the availability and use of opal suppressor tRNAs to enhance mycoplasma gene expression, their limited and variable efficiency (Brown et al., 1990; Raftery et al., 1984) raises questions about their effectiveness in the expression of genes with numerous TGA triplets. The efficiency of the *trpT*176 allele to insert tryptophan at UGA codons has been demonstrated to be context dependent, with the efficiency at one site being reduced by two-thirds compared to another (Raftery et al., 1984). The effectiveness of suppressor readthrough versus termination of protein synthesis depends upon a number of poorly defined factors such as the nature of the suppressor tRNA, messenger RNA context effects, interaction with other tRNAs on the ribosome, and competition with the release factor (Brown et al., 1990).

We sought to enhance the efficiency of the *trpT*176 allele by combining it with other mutations that also facilitate suppression of UGA codons due to their influence on termination events. The *prfB*3 mutation (Kawakami et al., 1988) is responsible for a single amino acid change in the *E. coli* peptide chain release factor 2 (Mikuni et al., 1991). RF-2 binds to the ribosome, recognizes UGA and UAA termination codons and facilitates termination of protein synthesis by triggering the release of the nascent polypeptide (Caskey et al., 1984; Weiss et al., 1984). It has been postulated that the *prfB*3 mutation leads to abnormal ribosomal stalling which is responsible for misreading of UGA codons (Gavini and Pulakat, 1991; Mikuni et al., 1991; Roesser et al., 1989). Since release factors compete with suppressor tRNAs for recognition of termination codons (Beaudet and Caskey, 1970; Ganoza and Tomkins, 1970; Martin et al., 1988), we postulated that a mutation that hinders RF-2 activity may provide the suppressor tRNA with a competitive advantage and might augment the insertion of tryptophan by *trpT*176 at UGA sites.

In addition to the role of the release factor, ribosomal RNA involvement has also been implicated in translational termination (Murgola et al., 1988; Prescott et al., 1991b; Shine and

Dalgarno, 1974). We have investigated the potential usefulness of a 16S rRNA mutation consisting of a deletion in base C1054 in the readthrough of UGA codons. Since this mutation was described by Prescott et al. as a "super suppressor" that enhances the activities of tRNA suppressors at nonsense codons (Prescott et al., 1991a), we anticipated an increase in the efficiency of *trpT*176 in this genetic background.

In order to explore the feasibility of our expression system, UGA suppressor strains were employed in the expression of portions of the P1 structural gene from *M. pneumoniae*. *M. pneumoniae*, the etiological agent of primary atypical pneumoniae in children and young adults, colonizes the mucosal surface of the respiratory tract (Jacobs, 1991). The P1 protein is a hydrophobic integral membrane protein which clusters at the tip of an attachment organelle and mediates cell adherence to the ciliated respiratory epithelium (Baseman et al., 1982; Feldner et al., 1982; Hu et al., 1977, 1982, 1983; Leith et al., 1983). The P1 adhesion typifies proteins that might be discovered using our system; it is a critical virulence determinant, as well as a major immunogen, which stimulates antibody production in experimental animals and human patients (Hu et al., 1982, 1983; Leith et al., 1983). The P1 gene, which has been cloned and sequenced (Inamine et al., 1988; Su et al., 1987), encodes 21 UGA codons. Early attempts to express the P1 protein in *E. coli* met with limited success (Dallo et al., 1988; Inamine et al., 1988, 1990; Schaper et al., 1987), and cloning was accomplished by using an approach that did not require complete protein expression (Inamine et al., 1988; Su et al., 1987). Based upon sequence information, we subcloned a fragment that contained four dispersed TGA triplets that encoded a 72 kDa protein. We also attempted expression of the entire P1 gene with or without the presence of its signal sequence. Protein expression from all constructions was monitored via immunoblot with monospecific, polyclonal rabbit antisera to the full-length P1 protein.

RESULTS AND DISCUSSION

Subcloning of *trpT*176

We established regulation of *trpT*176 by subcloning it downstream of the strong IPTG-inducible *trc* promoter on pTrc99 (Fig. 1). An adjacent *lacI*^{q} allele minimized basal expression of the suppressor activity and delayed the potentially deleterious effects of readthrough of the termination signals encoded in the host genome until the time of induction. The *lacI*^{q} and *trpT*176 alleles were inserted into plasmid pACYC184 to provide an origin of replication and an antibiotic resistance marker that were compatible with pMB1-derived cloning vectors. The resulting construction, pISM3001, displayed inducible suppression of a *lacZ(UGA)* mutation, as demonstrated by βGal assay (data not shown).

Enhancement of Opal Suppressor Efficiency

We investigated the feasibility of using *trpT*176 in conjunction with other mutations that exhibit UGA readthrough in an attempt to enhance suppressor efficiency. The plasmid pISM3001 was introduced into *Escherichia coli* strains YN2980 [*leu*(UGA) *lacZ*659(UGA) *trpA*9605(UAG) *his29*(UAG) *ilv thyA metB argH rpoB rpsL prfB*3] (Kawakami et al., 1988) and MDA6650 {*rrsB*(SuUGA-ΔC1054) *glyV*55 *Δ*(*tonB trpAB*17) [F' *trpA*(UGA211)]} (Murgola et al., 1988) via electroporation with a BTX Electro Cell Manipulator 600 according to manufacturer's instructions. Strain YN2980 contained the *prfB*3 mutation which impairs RF-2 activity. MDA6650 contained a single base deletion in its 16S rRNA. The resulting strains were viable, although the strain containing *prfB*3 grew poorly when *trpT*176 was induced. The plasmid pISM3001 was also introduced into JM105 {*Δ*(*lac-proAB*),*thi, rpsL, endA, sbcB*15, *hsdR*4(r_K^- m_K^+) [F', *traD*36, *proAB*, *lacI*^{q}, *lacZΔ*M15]}(Yanisch-Perron et al., 1985), a standard suppressor-free cloning host.

Subcloning of Mycoplasma Gene Sequences Containing TGA Triplets

The P1 gene encodes 21 UGA codons, many of them clustered in the center of the transcript. Based upon sequence information, we subcloned a portion of the P1 gene that contained four dispersed TGA triplets encoding a 72 kDa protein (Fig. 2). Hydrophilicity plots of the protein sequence had predicted several antigenic sites in this region which might be used to detect protein products with antisera (Su et al., 1987). Epitope mapping of overlapping peptides using the sera of infected patients had also identified some immunodominant antigenic domains within the cloned fragment (Jacobs et al., 1990). A linker containing an in-frame translational initiation triplet was used to position the fragment downstream of the RBS and the promoter in the prokaryotic expression vector pKK223-3.

The entire P1 gene, with or without its signal sequence, was also subcloned into pKK223-3 (Fig. 3). Upstream PCR primers containing in-frame translational initiation triplets and *Eco*RI restriction sites were utilized to amplify fragments that could be positioned at an optimal distance from the RBS and the promoter in plasmid pKK223-3 (Fig. 4). The first 59 amino acids of the P1 protein appear to compose a leader peptide, but since the steps involved in processing the P1 protein have not been elucidated, we were uncertain whether the signal peptide would be cleaved in an *E. coli* host. Inefficient processing might lead to the generation of several intermediate forms with different molecular weights. If the P1 protein translocation signals were recognized in *E. coli*, insertion into the membrane might be lethal to the cell. Therefore, alternate primers were used to selectively amplify sequences containing or lacking the signal sequence (Fig. 4).

Analysis of Expression System Efficacy

Plasmids carrying mycoplasma sequences were introduced into *E. coli* strains YN2980, MDA6650 and JM105 with or without the *trpT*176-containing plasmid pISM3001. Cells were

grown under inducing and noninducing conditions and expression of mycoplasma proteins was monitored via immunoblot with monospecific, polyclonal rabbit antisera to the full-length P1 protein (kindly provided by Joel B. Baseman).

Depending upon the number of termination codons that are suppressed, five polypeptides with molecular weights of 2, 15, 21, 60 and 72 kDa are expected to be produced from pISM3005 (Fig. 6). Strains YN2980 and MDA6550 both produced a strong band at approximately 60 kDa (Fig. 5, Panel A, lanes 2 and 3) under inducing conditions when transformed with plasmids pISM3001(*trpT*176) and pISM3005 (partial P1 gene). A less intense band near 72 kDa was also observed. This suggests efficient suppression of three UGA codons and at least a detectable level of readthrough of the fourth termination signal in a portion of the transcripts. A band which might correlate with the 21 kDa product was seen in strain MDA6650, although the other predicted products were not observed. Suppression efficiencies might have been sufficient to prevent the accumulation of detectable levels of the other predicted products. Alternatively, the antisera may not have recognized these products. Additional bands seen under inducing conditions (56, 51, and 38 kDa) could not be correlated with expected products; they might represent degradation products or result from translational reinitiation from sites downstream of UGA codons. Strain JM105, which lacks opal suppressor activity, produced a 63 kDa product in the presence of pISM3001. The inducible promoters on plasmids pISM3001 and pISM3005 did not appear to be tightly regulated in the JM105 genetic background because a 63 kDa protein product from plasmid pISM3005 was observed under both uninduced and induced conditions (Fig. 5, Panel A, lane 1). The control strains containing the vector pKK223-3 lacking P1 gene sequences produced no detectable protein products. Also, strains YN2980 and JM105 failed to generate detectable products in the absence of the *trpT*176 allele (data not shown). Strains YN2978 and YN2979, harboring *prfB*1 and *prfB*2 mutations respectively (Kawakami et al., 1988), displayed results similar to those seen with the *prfB*3 mutation (data not shown) and were not considered further.

Strains YN2980 and MDA6650, harboring plasmids pISM3001 (*trpT*176) and pISM3011 (P1 coding sequence including the signal sequence), produced a protein of approximately 40 kDa under inducing conditions (Fig. 5, Panel B, lanes 2 and 3). If the signal peptide is processed in *E. coli*, a 40 kDa product would indicate readthrough of 6 UGA codons. Alternatively, if the signal peptide is not removed, a 40 kDa product would indicate readthrough of 3 UGA codons. Strain JM105 produced no detectable products (lane 1).

Strain YN2980, harboring pISM3001 (*trpT*176) and pISM3012 (the P1 coding sequence lacking the signal sequence), generated a weak band at approximately 65 kDa, a doublet near 59 kDa and strong bands close to 54, 37, and 34 kDa (Fig. 5, Panel B, lanes 5 and 6). The 34 and 37 kDa products were also seen in strain MDA6650. Strain JM105 produced no detectable products with these plasmids (lane 4). Due to experimental variation in the molecular weight estimations and the fact that many of the predicted sizes are in close proximity, it is difficult to correlate observations with predictions (Fig. 6). The best estimate is that the doublet at 59 kDa represents the 63 kDa and 64 kDa predicted products, the observed 65 kDa band would then correlate with the predicted 66 kDa product and the observed 54 kDa band would correlate with the predicted 52 kDa product. The other bands would correlate with predicted products in the 33 - 39 kDa range. If these estimations are correct, readthrough of 9-10 UGA codons in YN2980 and 4-6 UGA codons in MDA6650 was demonstrated.

Strains that generated immunopositive products under inducing conditions usually produced a slight signal under noninducing conditions as well (Fig. 5). Apparently the use of rich media, although necessary to obtain reasonable growth of strains carrying both the *prfB*3 and the *trpT*176 alleles, engenders a certain amount of promoter leakiness.

CONCLUSIONS

(1) The combination of an opal suppressor tRNA (*trpT*176) with an additional mutation which posseses suppressor activity increases the efficiency of translational readthrough at UGA codons as compared to *trpT*176 alone. This is evidenced by the enhanced expression of P1 sequences in the presence of the *trpT*176 allele in strains YN2980 (*prfB*3) and MDA6650 {rrsB(SuUGA-ΔC1054)} as compared to strain JM105 (Fig. 5).

(2) Readthrough of 9-10 UGA codons has been demonstrated with this system. Suppression efficiency might be even greater with other mycoplasma sequences depending upon the distribution of the UGA codons within the gene transcript and the context surrounding each codon.

(3) Differences were observed between the *prfB*3 and the *rrsB*(SuUGA-ΔC1054) mutations in the expression of the cloned P1 sequences. These differences probably reflect the particular mechanism of suppression in each case. The *prfB*3 mutation produces an aberrant release factor protein that participates ineffectively in translational termination (Mikuni et al., 1991). The *rrsB*(SuUGA-ΔC1054) mutation contains a one base pair deletion in the 16S rRNA sequence (Murgola et al., 1988) that results in suppression of any termination codon in the presence of the appropriate suppressor tRNA (Prescott et al., 1991a). In both cases, the interaction of the suppressor tRNA with the translational complex is apparently enhanced. The context surrounding any specific UGA codon might have varying affects on suppression efficiency in the two different genetic backgrounds.

(4) The system has potential application in the screening of mycoplasma gene expression libraries. An *E. coli* cloning host that disregards the UGA termination signals within mycoplasma coding sequences would simplify the gene isolation process and could allow the detection of genes that might never be observed in a nonsuppressing genetic background.

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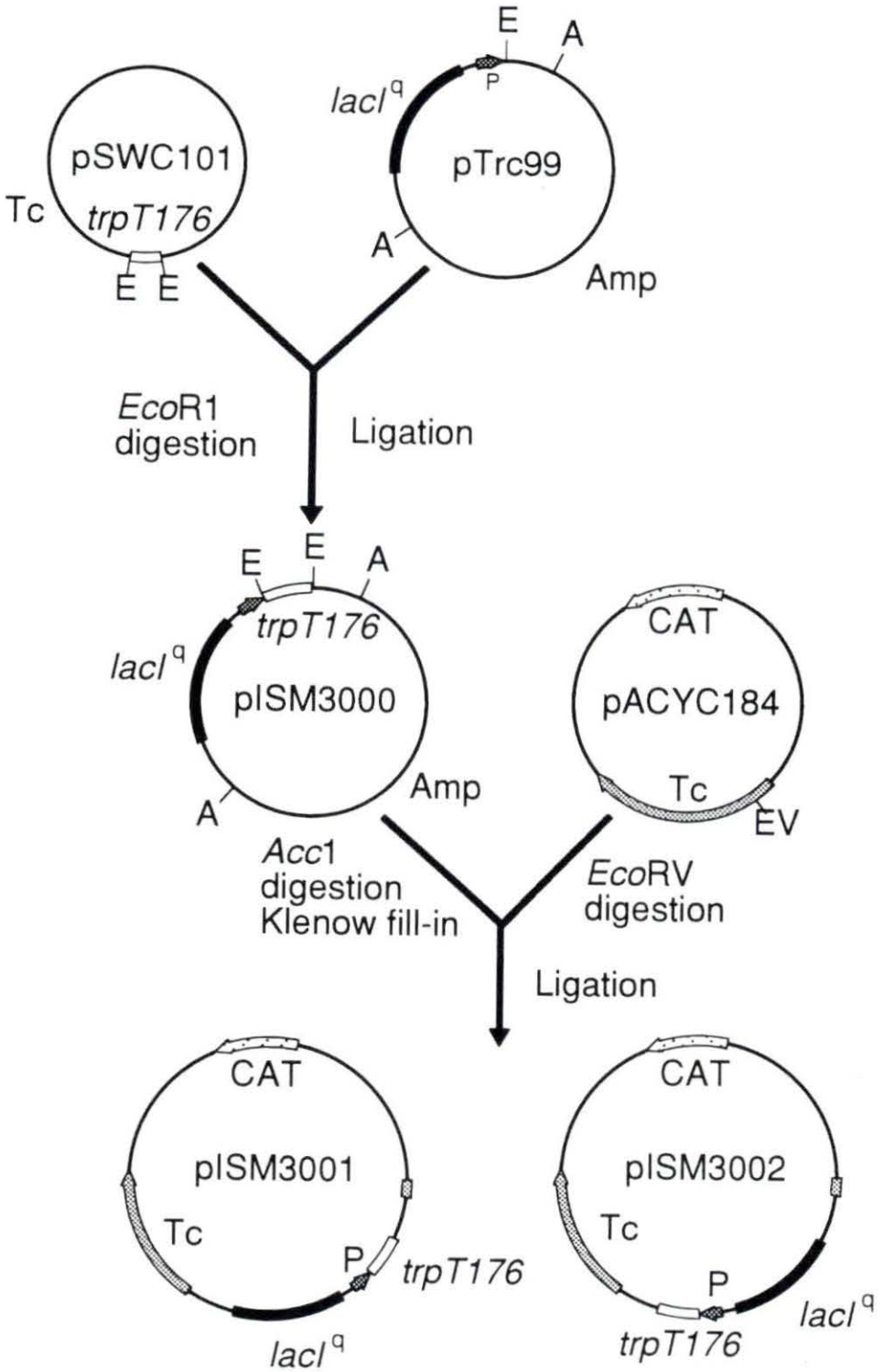
Fig. 1. Construction of plasmid pISM3001. Plasmid pSWC101 (Raftery et al., 1984) was digested with *Eco*RI to liberate a 0.3 kb fragment containing the *trpT176* allele. The fragment was isolated by electrophoresis in 1 x TAE buffer on a 2% New Sieve (FMC BioProducts, Rockland, ME) agarose gel and was recovered from a trough cut into the agarose followed by phenol/chloroform extraction and ethanol precipitation. The fragment was used in a ligation reaction with a plasmid mixture containing *Eco*RI restricted pTrc99A, pTrc99B, and pTrc99C (Amann et al., 1988). The ligation mixture was introduced into strain MY241[*lacZ659(UGA)*] (Raftery et al., 1984), and a functional opal suppressor plasmid, pISM3000, was isolated by screening on LB agar containing XGal and IPTG. Suppressor activity was confirmed by a βGal assay (Miller, 1972) (data not shown). Plasmid pISM3000 was digested with *Acc*I and was treated with Klenow to generate blunt ends. A 2.0 kb fragment containing the *trpT176* and *lacI*^{q} alleles was ligated into the *Eco*RV site of pACYC184 (Chang and Cohen, 1978) to generate plasmids pISM3001 and pISM3002, containing the fragment in opposite orientations. Only pISM3001 exhibited opal suppressor activity in a *lacZ659(UGA)* background as determined by a βGal assay (data not shown). The *trpT176* allele is denoted by the white bar. The *lacI*^{q} allele is denoted by the black bar. Directions of transcription are denoted by the arrows. Abbreviations: **A**, *Acc*I; **E**, *Eco*RI; **EV**, *Eco*RV; **P**, promoter. Restriction enzymes were obtained from Gibco BRL (Gaithersburg, MD) and New England Biolabs (Beverly, MA) and were used according to the manufacturer's recommendations. DNA polymerase I, Klenow, T4 DNA ligase, and T4 kinase were purchased from Gibco BRL. Taq polymerase was obtained from Promega (Madison, WI). dNTPs were obtained from Amersham International (Amersham, UK). Geneclean was purchased from Midwest Scientific (Valley Park, MO). Cloning manipulations were performed according to standard protocols (Sambrook et al., 1989).


E
A
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lacI q
pTrc99
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pSWC101
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trpT176
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Ligation
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A
trpT176
lacI q
pISM3000
A
Amp
CAT
pACYC184
Tc
EV
Acc1
digestion
Klenow fill-in
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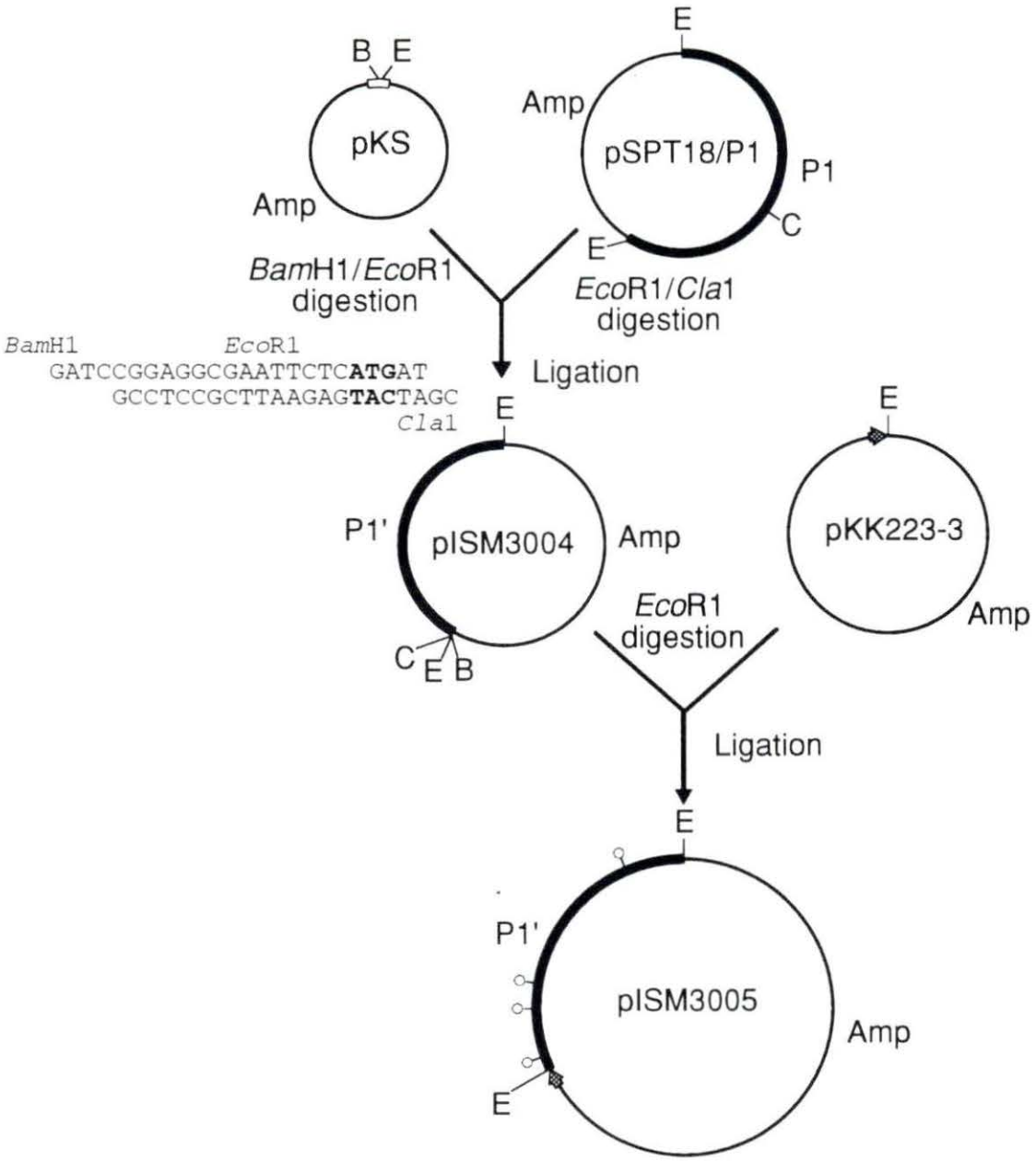
digestion
Ligation
CAT
pISM3001
Tc
P
trpT176
lacI q
CAT
pISM3002
Tc
P
lacI q
trpT176

Fig. 2. Construction of plasmid pISM3005. Plasmid pSPT18/P1, a derivative of plasmid pSPT18 containing the P1 coding sequence on an *Eco*RI fragment (R. Herrmann), was digested with *Eco*RI/*Cla*I/*Bam*HI, and a 2.7 kb *Eco*RI/*ClaI* fragment containing the 3' portion of the P1 gene was isolated on a 1.5% agarose gel in 1 x TAE buffer. The fragment was purified using Geneclean and was ligated into *Bam*HI/*Eco*RI restricted pKS(+) (Stratagene, La Jolla, CA) utilizing a 22 bp *Bam*HI/*ClaI* linker (Iowa State Nucleic Acid Facility). The linker was constructed such that an ATG initiation triplet (bold) was positioned in-frame with respect to the P1 gene fragment after ligation. The resulting construction, pISM3004, was digested with *Eco*RI and *Pvu*I (site not shown) to generate fragments that were resolvable by agarose gel electrophoresis, and a 2.7 kb EcoRI fragment containing the partial P1 gene and the ATG triplet from the linker were isolated from a 0.4% agarose gel in 1 x TAE buffer. The fragment was ligated into the *Eco*RI site of pKK223-3 (Pharmacia). The orientation of the fragment was confirmed by restriction digestion. The dark bar represents the P1 gene sequence. The positions of the TGA sequences in the P1 fragment are indicated by open circles.. The IPTG-inducible promoter is designated by the arrow. Abbreviations: **B**, *Bam*HI; **C**, *Cla*I; **E**, *Eco*RI.


B E
pKS
Amp
E
Amp
pSPT18/P1
P1
C
E
BamH1/EcoR1
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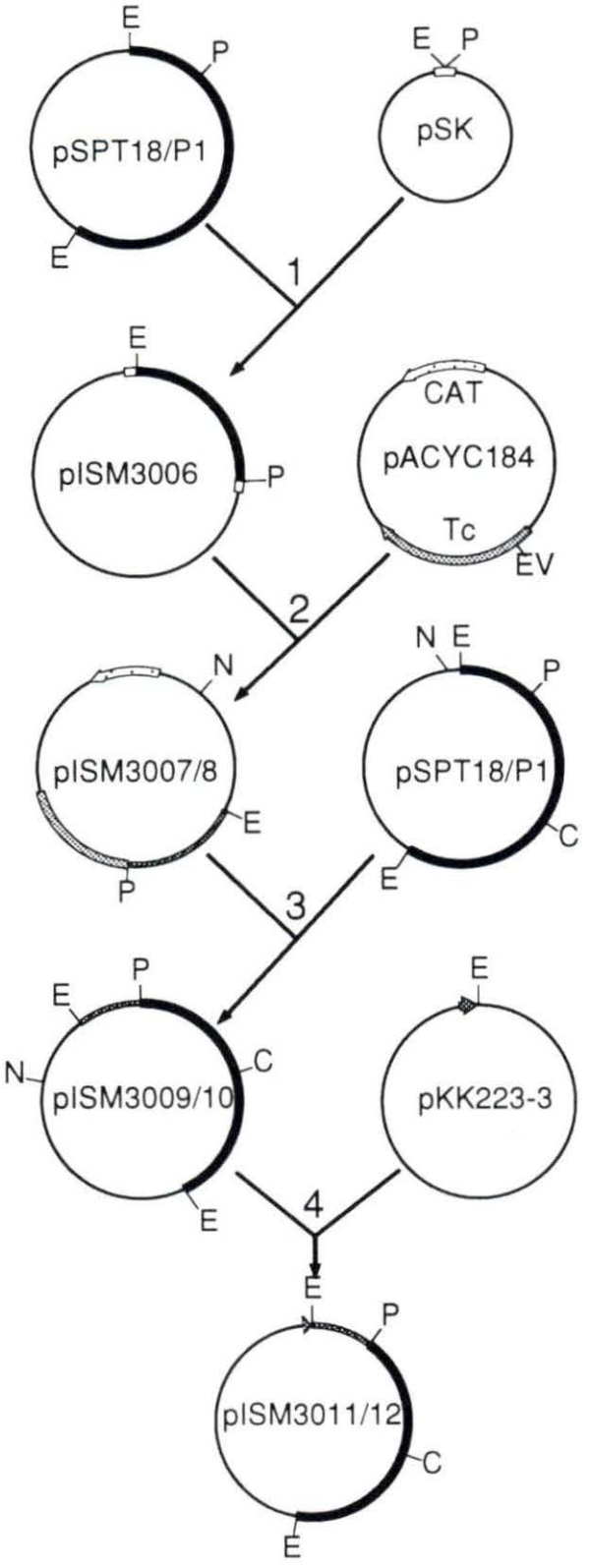
EcoR1/Cla1
digestion
BamH1 EcoR1
GATCCGGAGGCGAATTCTCATGAT
GCCTCCGCTTAAGAGTACTAGC
Cla1
Ligation
E
P1'
pISM3004
Amp
C E B
E
pKK223-3
Amp
EcoR1
digestion
Ligation
E
P1'
pISM3005
Amp
E

Fig. 3. Construction of plasmids pISM3011 and pISM3012. **1:** Plasmid pSPT18/P1 was digested with *Eco*RI /*Pst*I and a 1.1 kb fragment from the 5' portion of the P1 gene was ligated into *Eco*RI /*Pst*I restricted pSK (Stratagene) to generate plasmid pISM3006. **2**: Plasmid pISM3006 was used as a template in a PCR amplification (Fig. 4). Primers were constructed to selectively amplify the 5' portion of the P1 gene, with or without the signal sequence. An in-frame initiation triplet was included in each upstream primer. The ends of the PCR products were polished with DNA polymerase I and T4 kinase, and 1 kb and 0.8 kb fragments were individually ligated into the *Eco*RV site of pACYC184 to generate pISM3007 (with the signal sequence) and pISM3008 (without the signal sequence). **3**: These constructions were digested with *Nhe*I/*Pst*I and the 2.1 kb and 1.9 kb fragments, containing P1 sequences from pISM3007 and pISM3008, respectively, were inserted into pSPT18/P1 to replace the original 5' portion of the P1 gene. **4**: The resulting plasmids, pISM3009 and pISM3010, were digested with *Eco*RI, and the 5 kb and 4.8 kb P1 gene fragments from each were inserted behind the promoter in pKK223-3 to generate pISM3011 and pISM3012, respectively. Bands for ligation were isolated on 0.4% agarose gels in 1 x TAE buffer, and purified by Geneclean. Only relevant restriction enzyme sites are given. The arrows denote direction of transcription. Abbreviations: **C**, *Cla*I; **E**, *Eco*RI; **EV**, *Eco*RV; **N**, *Nhe*I; **P**, *Pst*I.


E
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pSPT18/P1
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pACYC184
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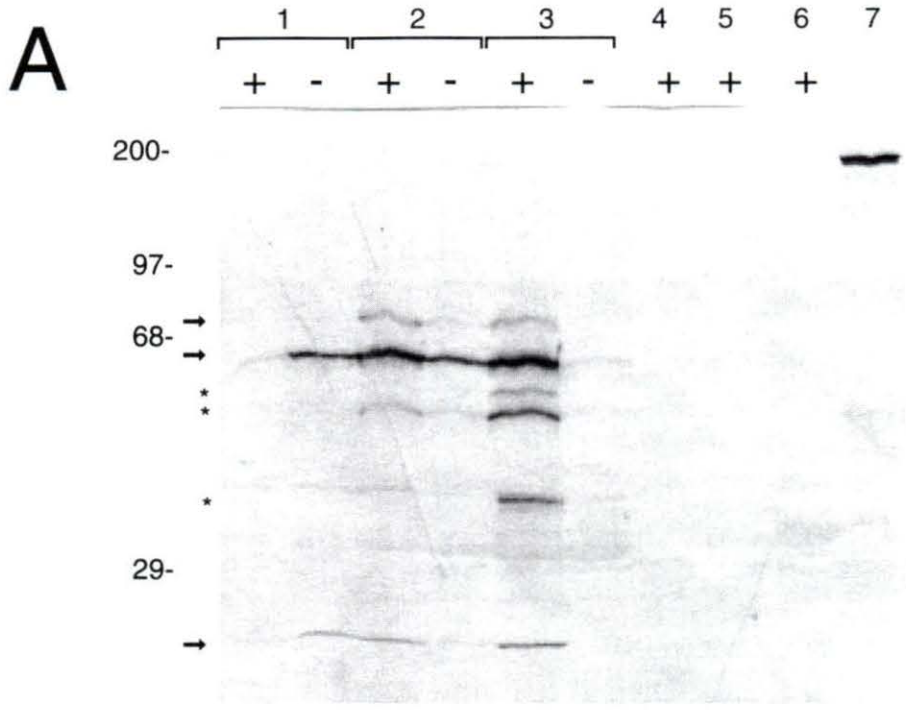
pSPT18/P1
C
E
3
E
P
E
N
pISM3009/10
C
E
E
pKK223-3
4
E
P
pISM3011/12
C
E

Fig. 4. Primers for PCR amplification of the 5' portion of the P1 gene, with or without the signal sequence, were synthesized by the Iowa State Nucleic Acid Facility. A 26 bp oligo that was homologous to the initiation ATG triplet and the beginning of the leader sequence was constructed with an *Eco*RI restriction recognition site (single underline) for proximal insertion behind a promoter. In order to delete the signal sequence, a 29 bp oligo with homology to the mature protein coding sequence was synthesized with an upstream *Eco*RI site (single underline) and an initiation ATG triplet (double underline). The asterisk indicates the beginning of the mature polypeptide. The T3 sequencing primer (Alting-Mees and Short, 1989) which has a recognition site in the pSK vector, was used as a downstream primer for both reactions. One hundred nanograms of each primer was used to amplify 50 ng of plasmid template DNA. Amplification reactions were performed in a Model 50 Tempcycler (Coy Laboratory Products, Inc., Ann Arbor, MI) with the following reaction conditions: denatured at 94°C for 2 min, annealed at 45°C for 1.5 min, polymerized at 72°C for 2.8 min, 1 cycle; denatured at 94°C for 1 min, annealed at 45°C for 1 min, polymerized at 72°C for 2 min, 25 cycles; denatured at 94°C for 1.5 min, annealed at 45°C for 1.5 min, polymerized at 72°C for 5 min, 1 cycle. Amplified products were isolated on a 0.4% agarose gel in 1 x TAE buffer and were purified by Geneclean.


EcoRI
GGGAATTCATGCACCAAACCAAAAAA
TACGTGGTTTGGTTTTTTTGACGGAACAGGTTCAGGTGAACCTAAGAGTAGGAG
TGGCGGTGGCGGAGGGAGCGCTGCCCTGAGTGGCATCACCCTGTGAAGTGTTCA
TGGTGGTGCTGCGAGTTCGCGGTCGTTAAATCGATGTGGGCGGGACTGCTCCAG
EcoRI *
GGGAATTCATGAATGCCATCAACCCGCGC
CGCGACGCGGTGTGGTTACGGTAGTTGGGCGCG

Fig. 5. Analysis of opal suppressor efficiency. Strains to be analyzed were grown with shaking overnight at 37°C in 5 ml superbroth (32 g/L Bacto tryptone, 20g/L yeast extract, 5g/L NaCl) with the appropriate antibiotics. The following antibiotics were used at the indicated concentrations: tetracycline, 12.5 μg/ml; chloramphenicol, 20 μg/ml; ampicillin, 50 μg/ml. (Sigma Chemical Company, St. Louis, MO). One milliliter of each overnight culture was harvested by centrifugation, resuspended in 5 ml fresh superbroth media containing antibiotics and incubated at 37°C for 4 h. Induced cultures were subcultured with 1 mM IPTG. Strains containing pISM3001 were grown in duplicate under inducing conditions to ensure adequate protein yield. Cultures were harvested by centrifugation and were resuspended in water. Protein assays were done using BioRad protein assay reagent. Fifty micrograms of each protein sample was precipitated overnight at -20°C with 10 volumes ethanol, centrifuged at 12,000 rpm, disrupted 1:1 with denaturing buffer (Laemmli, 1970), and boiled for 5 minutes. Samples were analyzed on 7.5%-15% gradient SDS-PAGE gels according to the method of Laemmli (Laemmli, 1970). Proteins were transferred to Westran PVDF membranes (Midwest Scientific, Valley Park, MO) according to the method of Towbin (Towbin et al., 1979). Molecular weight standards were obtained from Gibco BRL (Gaithersburg, MD) and were used according to the manufacturer's instructions. The membranes were air dried at room temperature for 10 min, and the molecular weight standards were stained with India ink (Pelikan, Hannover, West Germany). The sample lanes were blocked with PBS - 5% Carnation nonfat dry milk - 0.05% Tween 20 - 0.02% thimersol for 1 h at room temperature and were then incubated overnight at 25°C with a 1:3000 dilution of monospecific, polyclonal rabbit antisera to the P1 protein obtained from J. Baseman. The membranes were washed four times (15 min each) with blocking solution. The membranes were incubated for 1.5 h at room temperature with goat anti-rabbit alkaline phosphatase conjugate (Cappel, Chester, PA) at a dilution of 1:1000 in blocking solution. The membranes were given five washes (15 min each) with blocking solution, TS, TS, TSN and TS, respectively. Napthol AS-MX phosphate (0.75 mg/ml) and Fast Red (1.5 mg/ml) in 20 mM Tris (pH 7.5) (Sigma) were utilized for development. The immunoblots were digitized using a Hewlett Packard ScanJet IIc digitizer, and the lane and molecular weight markers were added using MacDraw Pro (Macintosh). (+) indicates IPTG induced cultures, (-) indicates uninduced cultures. (**Panel A**) Expression of a subcloned fragment from the P1 gene (pISM3005) in *E. coli*;; lanes: **1**, JM105 (pISM3001, pISM3005); **2**, YN2980 (pISM3001, pISM3005); **3**, MDA6650 (pISM3001, pISM3005); **4**, JM105 (pISM3001, pKK223-3); **5**, YN2980 (pISM3001, pKK223-3); **6**, MDA6650 (pISM3001, pKK223-3); **7**, *M. pneumoniae* (30 μg protein). (**Panel B**) Expression of the P1 gene with and without the signal sequence in *E. coli*; lanes: **1**, JM105 (pISM3001, pISM3011); **2**, YN2980 (pISM3001, pISM3011); **3**, MDA6650 (pISM3001, pISM3011); **4**, JM105 (pISM3001, pISM3012); **5**, YN2980 (pISM3001, pISM3012); **6**, MDA6650 (pISM3001, pISM3012).


A
1
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+ - + - + - + + +
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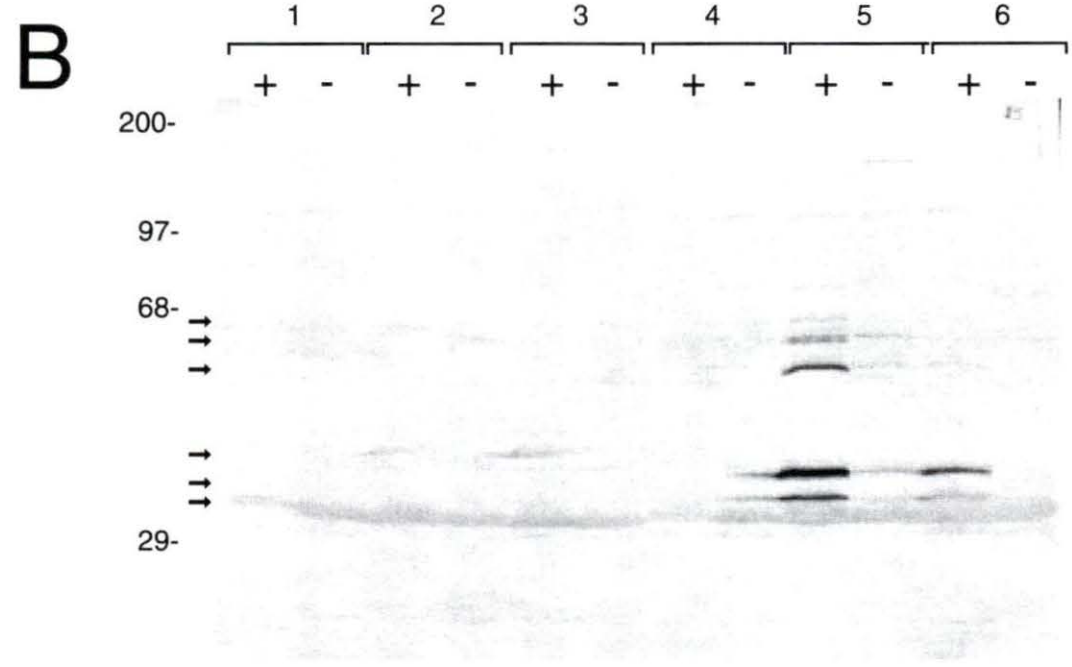
B
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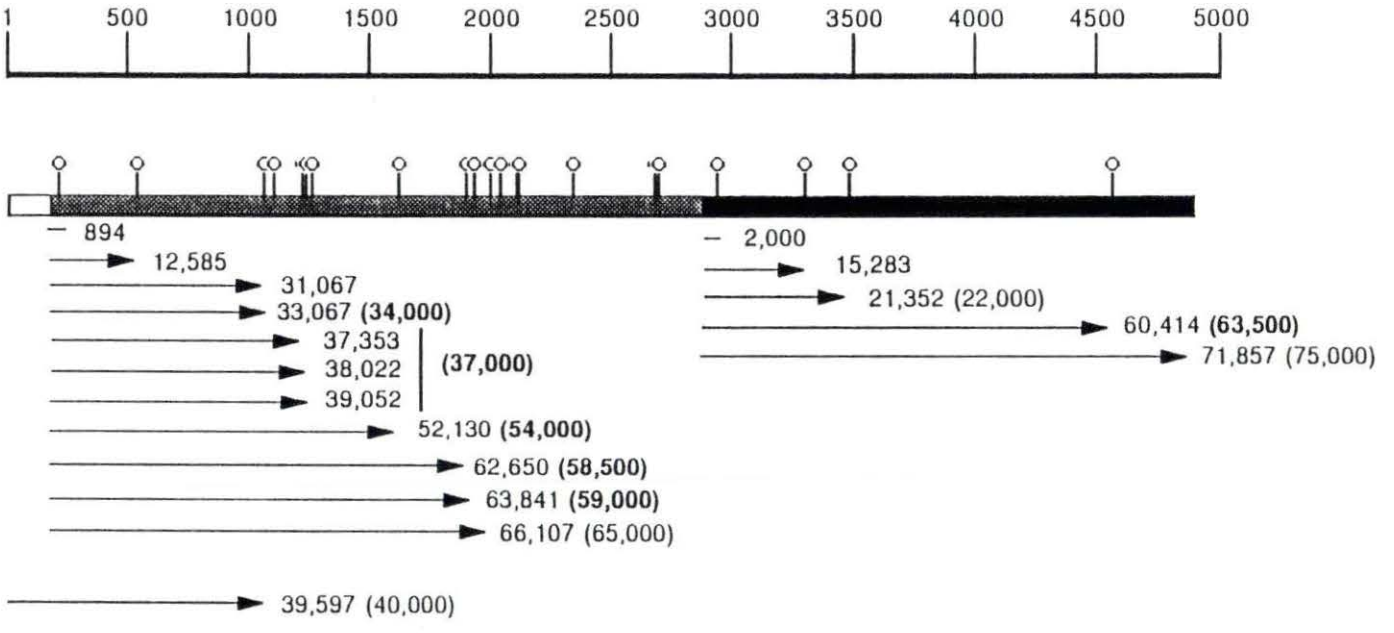
Fig. 6. Predicted polypeptides resulting from premature translational termination in the P1 open reading frame. The fragment represents the coding sequence of the P1 structural gene (Su et al., 1987). The open bar represents the signal sequence, and the solid black bar represents the DNA sequence cloned into plasmid pISM3005. The open circles represent the 21 TGA triplets within the 4481 nt sequence and thus potential translational termination sites. The arrows represent potential translational products from three different plasmid constructions. The predicted molecular weight is given for premature terminated products, and the estimated molecular weight of the corresponding immunoreactive protein band (Fig. 5) is given in parentheses. Bold indicates a strongly reactive band.


1
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
894
12,585
31,067
33,067 (34,000)
37,353
38,022
39,052
(37,000)
52,130 (54,000)
62,650 (58,500)
63,841 (59,000)
66,107 (65,000)
39,597 (40,000)
2,000
15,283
21,352 (22,000)
60,414 (63,500)
71,857 (75,000)

PAPER 2. IDENTIFICATION OF *MYCOPLASMA HYOPNEUMONIAE* ANTIGENS IN *ESCHERICHIA COLI* USING OPAL SUPPRESSOR MUTATIONS FOR ENHANCED EXPRESSION

Identification of *Mycoplasma hyopneumoniae* antigens in *Escherichia coli* using opal suppressor mutations for enhanced expression

by

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ABSTRACT

M. hyopneumoniae is the causative agent of an economically significant swine mycoplasmal pneumonia. Although *E. coli* cloning hosts have been used to identify gene products with diagnostic or vaccine potential, there have been suggestions that an unusual codon usage pattern, which is characteristic of many mycoplasma species, might preclude the isolation of some *M. hyopneumoniae* genes. Most mycoplasmas use the UGA termination codon to encode the amino acid tryptophan. Premature truncation of cloned proteins places limitations on the applicability of *E. coli* cloning hosts for the screening of mycoplasma genomic libraries. We have developed a cloning system for the expression of mycoplasma genes in *E. coli* that facilitates the insertion of tryptophan at UGA sites via the combination of an opal suppressor tRNA (*trpT*176) with a mutation in the release factor 2 gene (*prfB*3). We have employed this system in the screening of lambda libraries containing gene sequences from *M. hyopneumoniae*.

INTRODUCTION

M. hyopneumoniae is the etiological agent of mycoplasmal pneumoniae in swine. Despite a low incidence of mortality, this disease has a significant economic impact due to diminished feed conversion and enhanced susceptibility to secondary bacterial infections in infected animals (1, 28, 36). Development of specific diagnostic tests and an efficacious vaccine for these small cell wall-less prokaryotes has been hindered by their serological resemblance to other nonpathogenic swine commmensals (6, 38) and by a paucity of genetic and biochemical analyses. These organisms grow poorly in vitro, even in complex serum-rich media, and traditional gene transfer techniques are not applicable due to difficulties in transformation and a lack of suitable cloning vectors.

E. coli cloning hosts have been used to express *M. hyopneumoniae* gene sequences in an attempt to circumvent the obtacles which are encountered when studying this organism. Immunological screening of gene libraries in *E. coli* has been used to isolate epitopes with diagnostic or vaccine potential (1, 16, 17, 32, 37). Protein expression in *E. coli* also has potential in the large scale production of antigens for scientific analysis or commercial applications. Although this approach has facilitated the identification and characterization of certain immunogenic peptides, there have been suggestions that *M. hyopneumoniae* (10, 17) utilizes the unusual codon usage pattern which is found in most other mycoplasma species (3, 4, 5, 9, 14, 18, 25, 39). Many mycoplasmas utilize the UGA termination codon to encode the amino acid tryptophan. This results in the premature truncation of many mycoplasma proteins when they are expressed in *E. coli* and may prevent their detection in cases where the partial protein products do not contain a crucial epitope or lack functional activity (4, 5, 8, 13, 22, 30, 33).

We have previously described the development of a cloning system for the enhanced expression in *E. coli* of mycoplasma sequences which direct the incorporation of tryptophan

via the use of UGA codons (31). We have demonstrated readthrough of 9-10 UGA codons in a transcript encoding the P1 adhesion from *M. pneumoniae* by combining an opal supppressor tRNA *(trpT*176) with the *prfB*3 mutation, which enhances tryptophan insertion by altering the effectiveness of the peptide chain termination factor, release factor-2. In this paper, we analyze the efficacy of this system in the expression of lambda libraries containing gene sequences from *M. hyopneumoniae*.

MATERIALS AND METHODS

Enzymes and Chemical Reagents

Restriction enzymes were obtained from Gibco BRL (Gaithersburg, Md.) and New England Biolabs (Beverly, Mass.) and were used according to the manufacturer's recommmendations. T4 DNA ligase and Klenow fragment were purchased from Gibco BRL. dNTPs were obtained from Amersham International (Amersham, UK). Cloning manipulations were performed according to standard protocols (29). Gigapack Packaging Extracts were supplied by Stratagene Cloning Systems (La Jolla, Cal.). XGal and IPTG were obtained from Gold Biotechnology (St. Louis, Mo.). Antibiotics, Napthol AS-MX Phosphate and Fast Red were purchased from Sigma Chemical Company (St. Louis, Mo.). HRP Color Development Reagent was obtained from Bio-Rad Laboratories (Richmond, Cal.). Alkaline phosphatase and Horseradish peroxidase conjugates were supplied by Cappel (Chester, Penn.).

Bacterial Strains and Growth Conditions

The *E. coli* strains used in this study are listed in Table 1. The chloramphenicol resistant plasmid pISM3001 contains the *lacI*^{q} allele and an inducible opal suppressor allele (*trpT*176) in plasmid pACYC184 (31). Strain XL1-Blue was grown overnight in Luria broth media (10 g/L Bacto tryptone, 5 g/L yeast extract, 5 g/L NaCl) with 0.2% maltose and 12.5 µg/ml tetracycline. The cells were harvested by centrifugation and were resuspended in 10 mM $MgSO_4$ to an optical density at 600 nm of 0.6. Lawns were made with 200 µl of cells and 3 ml of Luria broth top agar (0.7% agar) which were poured onto phage plates (Luria agar with 2.5 mM $CaCl_2$). Strain ISM586 was grown overnight in superbroth (32 g/L Bacto tryptone, 20 g/L yeast extract, 5 g/L NaCl) media containing 0.2% maltose, 12.5 µg/ml tetracycline, and 30 µg/ml chloramphenicol. The cells were harvested by centrifugation and were resuspended in superbroth with 10 mM $MgSO_4$ to an optical density at 600 nm of 1.0. Lawns were made with

1 ml of cells and 2 ml of superbroth top agar (1% agar) containing 20 µg/ml chloramphenicol, which were poured onto superbroth phage plates. Strain LE392 was grown overnight in Luria broth media with 0.2% maltose. The cells were harvested by centrifugation and were resuspended in 10 mM $MgSO_4$ to an optical density at 600 nm of 0.5. One hundred µl of cells were plated with 3 ml of Luria broth top agar, which were poured onto phage plates. Strains ISM666, ISM667, and ISM668 were grown with the appropriate antibiotics, harvested, and 150 µl (ISM666 and ISM667) or 200 µl (ISM668) of cells were plated following the same procedure as LE392, with the addition of 20 µg/ml chloramphenicol in the top agar. All bacterial growth was conducted at 37°C.

Construction of Lambda Libraries

Construction of Lambda ZAP II library

Chromosomal DNA from *M. hyopneumoniae* strain J was partially digested with *Sau*3A1 and was size-fractionated via sucrose gradient centrifugation (19). The fractions containing fragments larger than 8 kb, as determined by agarose gel electrophoresis, were pooled and treated with Klenow and dGTP/dATP in a partial fill-in reaction. Lambda Zap II DNA was digested to completion with *Xho*I and was partially filled-in with Klenow and dTTP/dCTP. Two hundred ng of chromosomal DNA was ligated with 1 µg of Lambda Zap II vector. A portion of the ligation mixture was packaged in vitro with Gigapack Packaging Extract according to the manufacturer's instructions. The library was plated on strain XL1-Blue, utilizing top agar containing 4 mg/ml Xgal and 2.5 mM IPTG.

Approximately 1000 white plaques were dispensed into microtiter plates containing 200 µl SM buffer (5.8 g/L NaCl, 2.0 g/L $MgSO_4.7H_2O$, 2 g/L gelatin in 50 mM Tris pH 7.5) per well and were stored overnight at 4°C to allow the phage to diffuse out of the plugs. Ten µl of each plug supernatant was used to inoculate a "mini-lawn" in another microtiter plate well. Each lawn was prepared with 100 µl Luria broth bottom agar and 50 µl Luria broth top agar

containing XL1-Blue cells. After the lawns cleared, phage lysates were harvested with 100 µl of SM buffer by shaking at 4°C for 2-4 h. Ten µl of chloroform was added to each well, and the plates were centrifuged at 500 x *g* for 10 min. Fifty µl of each supernatant was diluted into 50 µl of SM buffer in fresh microtiter plates which were stored at 4°C until the time of screening.

Construction of Lambda FIX library

Chromosomal DNA from *M. hyopneumoniae* strain J was partially digested with *Sau*3A1 and was size-fractionated via sucrose gradient centrifugation (19). The fractions containing 9-22 kb fragments, as determined by agarose gel electrophoresis, were pooled and ligated into the Lambda Fix cloning vector according to the manufacturer's instructions. A portion of the ligation mixture was packaged in vitro using packaging mixes produced from strain SMR10, and recombinant phage were selected by plating on the *E. coli* P2 lysogen strain P2392. Approximately 1,200 independent recombinant phage were obtained and amplified in *E. coli* strain JM105. The amplified lysate was frozen in 1 ml aliquots at -70°C until the time of screening. Library construction and amplification was done according to the manufacturer's instructions.

Lambda Library Screening

Screening of Lambda ZAP II recombinant library

A replicator pin was used to transfer phage from microtiter plates to bacterial lawns, and cells were grown for 3 h (XL1-Blue) or 4 h (ISM586) before the application of IPTG-saturated transfer membranes (Micron Separations Inc., Westboro, Mass.). Plates were incubated overnight and were cooled at 4°C for 30 min before removal of the membranes. Membranes were washed for 30 min in TS buffer (10 mM Tris, 0.9% sodium chloride, pH 7.4) with 0.05% Tween 20 (TST), and nonspecific binding sites were blocked with TST, 5% Carnation

nonfat dry milk, 0.02% thimersol (blocking solution) for 1h. Membranes were incubated overnight at 4°C with a 1:400 dilution of anti-*M. hyopneumoniae* swine antiserum (kindly provided by Dr. R. F. Ross) in blocking solution. This antiserum, referred to as immune swine serum S195, was prepared in the following way. Swine were immunized intramuscularly twice with lung homogenate containing *M. hyopenumoniae* in Freundt's incomplete adjuvant (1:2) at a two week interval. Seventeen days after the second injection, the swine were challenged intratracheally with 10 mls of lung homogenate. Serum was collected 40 days later, ELISA titers were determined, and western blots were performed to assess the quality of the antiserum. Membranes were washed three times (10 min each) in TST and were incubated for 2 h at 25°C with a 1:1000 dilution of goat anti-swine horseradish peroxidase conjugate in TST. After two (15 min) washes in TST and TS, HRP Development Substrate was applied.

Screening of Lambda FIX recombinant library

Strain LE392 was infected with approximately 150-200 pfu per plate and the lawns were incubated for 3 h prior to the addition of IPTG-saturated nitrocellulose transfer membranes. The plates were incubated for an additional 5 h to allow plaque formation and phage absorption to the membranes, after which the membranes were lifted and air dried in a sterile hood for 10 min. The membranes were transferred to lawns of strain ISM586 which had been growing for 3 h prior to membrane transfer. Fresh membranes were applied to the LE392 lawns after removal of the first set, and all plates were incubated overnight.

Plates were incubated without covers at 37°C for 30 min and were stored at -20°C for 5 min before the membranes were removed. Membranes were washed for 30 min in TST buffer, and nonspecific binding sites were blocked with blocking buffer for 30 min. Membranes were incubated for 3 h at 25°C with a 1:600 dilution of hyperimmune rabbit antiserum (R322) (kindly provided by Dr. R. F. Ross) in blocking solution. The antiserum was preabsorbed

with a lysate of LE392/XL1-Blue cells. Membranes were washed three times (10 min each) in TST buffer and were incubated for 1 hr at 25°C with a 1:1000 dilution of goat anti-rabbit alkaline phosphatase conjugate in blocking solution. After a second series of washes, Napthol AS-MX Phosphate (0.75mg/ml) and Fast Red (1.5mg/ml) in 20 mM Tris (pH 7.5) were utilized for development.

Analysis of Immunoreactive Recombinant Lambda Clones

Positive plaques were streaked for isolated plaque formation on a lawn of strain LE392 and single plaques were picked into 1 ml of SM buffer. High titer phage stocks were subsequently made by the plate lysis procedure (19). Phage clones from both libraries, which were positive only when grown on strain ISM586, were analyzed further. Phage stocks were dispensed into a microtiter plate and were rescreened on strains LE392, ISM586, ISM666, ISM667, and ISM668 by inoculating fresh lawns with a replicator pin or by spotting 1 µl aliquots onto lawns. Strains were grown overnight prior to incubation with IPTG-saturated nitocellulose membranes for 4-5 hrs. Membranes were subsequently lifted and processed as described above. Membranes from all strains were screened with both swine immune (S195) and rabbit hyperimmune (R322) serum to *M. hyopneumoniae*. Lambda Zap II and Lambda Fix nonrecombinant vector controls were included on each membrane.

RESULTS

Screening of the Lambda Zap II Library

Approximately 1,000 lambda clones were grown under inducing conditions on the nonsuppressor strain XL1-Blue and the opal suppressor strain ISM586. Induction was necessary for the production of the *trpT*176 allele and for the expression of recombinant clones whose transcription depended upon the IPTG-inducible *lac* promoter within the Lambda ZAP II vector. Since strain ISM586 grows poorly under inducing conditions, the plates were incubated for several hours before induction, and a relatively large numbers of cells were grown in rich media in order to achieve an appropriate lawn density.

Plaque lifts were screened with swine antiserum raised to a lung homogenate containing *M. hyopneumoniae*. Only six positive plaques were identified, and they had all been grown on strain ISM586. Random clones were also screened for the presence of inserts by using helper phage to rescue the Bluescript plasmid from the vector according to manufacturer's instructions (data not shown). The resulting plasmids were analyszed by restriction enzyme digestion. Only one out of ten clones contained an insert, which explains the low frequency of positive plaques found during the immunological screening. Because of the low frequency of recombinants, the library was not studied further. However, the nature of those clones that were positive only on the suppressor strain was investigated.

Screening of the Lambda Fix Library

Approximately 1,400 clones were analyzed by growth on the nonsuppressor strain LE392 and the opal suppressor strain ISM586. Bacteriophage lambda grew at a relatively low efficiency on lawns of strain ISM586. Therefore, phage growth on the nonsuppressor strain was used to increase the titer of each clone such that it would result in significant clearing when

used to infect the suppressor strain. Nitrocellulose lifts from strain LE392 plates were used as a source of inoculum for strain ISM586.

Plaque lifts were screened with a rabbit hyperimmune antiserum raised against *M. hyopneumoniae*. Sixty-one clones were positive when grown on strain LE392. Although the plaques were tiny, most of those clones also appeared to be positive when grown on strain ISM586. There were sixteen additional clones which appeared to be positive during the initial screening only when grown on strain ISM586, but not when grown on strain LE392.

Analysis of Immunoreactive Recombinant Lambda Clones

Table 2 shows the results of screening the ISM586-positive recombinant lambda phage on different *E. coli* genetic backgrounds. Strains containing various suppressor mutations were used in an attempt to confirm the fact that UGA readthrough was responsible for the positive signals observed on strain ISM586. All of the suppressor strains carry plasmid pISM3001 containing the *trpT*176 allele which encodes a UGA suppressor $tRNA^{trp}$ (31). Strain ISM666 contains a chromosomal deletion of cytosine at position 1054 in the 16S rRNA sequence (21). Strain ISM667 contains a C to A transversion at position 1054 in 16S rRNA. The mutation is contained on a plasmid carrying the *rrsB* operon (12). Strain ISM668 is a ribosomal ambiguity (*ram*) mutant which contains an alteration in ribosomal protein S4 (2).

Of the six Lambda Zap II clones which were originally positive only on strain ISM586 using swine immune serum, four clones were also positive with rabbit hyperimmune serum when grown on strain ISM586. Two Lambda ZAP II clones were unusual in that they reacted only with the immune swine serum , but not with the rabbit hyperimmune serum, when grown on strain ISM586. Bluescript plasmids generated after in vivo excision of these lambda clones revealed identical restriction enzyme digestion patterns and harbored a fragment of *M. hyopneumoniae* chromosomal DNA 1.8 kb in size (data not shown). All six clones were negative on strain LE392 with both antisera.

Of the sixteen Lambda Fix clones which were originally positive only on strain ISM586 with rabbit hyperimmune serum, twelve were positive on strain ISM586 with both rabbit and swine sera. The remaining four clones were weakly positve with swine, but not with rabbit upon rescreening on strain ISM586. All of the clones were negative on strain LE392 with both antisera.

The results with strain ISM666 appeared to mirror those seen with strain ISM586, but the signals were often not as strong as those seen on strain ISM586, and they displayed varying intensities from one screening to another. However, six of the clones consistently showed positive signals with both antisera when grown on this strain. Strain ISM667 (data not shown) gave results which were similar to strain ISM666 with most lambda clones, but the signals were often weak and more variable from one screening to another, making it difficult to distinguish true positive clones. None of the ISM586-only positive clones were positive on strain ISM668 although two LE392 positive clones used as controls did produce positive signals on this strain when screened with the rabbit serum (data not shown).

DISCUSSION

We have demonstrated that an *E. coli* cloning host (strain ISM586), containing opal suppressor mutations, can be used to screen lambda libraries of mycoplasmal genomic DNA and can detect protein expression in clones which is not normally seen in nonsuppressor strains. The detection of additional clones in the suppressor background probably reflects the readthrough of UGA codons which encode tryptophan in mycoplasma sequences. The opal suppressor tRNA (*trpT*176) inserts the amino acid tryptophan at UGA sites in mRNA (24). The RF-2 protein normally binds to the ribosome at UGA and UAA termination codons and facilitates the release of the nascent polypeptide (7, 35). The *prfB*3 mutation alters the activity of RF-2 such that it is no longer an effective competitor with the opal suppressor tRNA and therefore enhances the efficiency of tryptophan insertion (11, 15, 20, 26). The observation that many of the clones generated positive signals on another suppressor strain (ISM666), further suggests that expression is probably a consequence of UGA suppression.

Strain ISM666 gave weaker and more variable signals than strain ISM586. This is probably due to different suppression mechanisms and corresponding varying sensitivities to context effects. Some clones were expressed almost as well on both strains. A previous comparison of strains ISM586 and ISM666 in the expression of the P1 adhesion from *M. pneumoniae* revealed similar differences in UGA suppression efficiencies (31). Depending upon the requirements of a particular situation, strain ISM666 may provide adequate UGA readthrough. This strain has superior growth characteristics than strain ISM586 and supports improved lambda growth. Although these features probably reflect a lower level of UGA suppression, they might prove useful in certain situations where less suppression is required.

Strain ISM667 did not perform as well as strain ISM666; signals were weak and variable. This is consistent with the fact that the C to A mutation displays a lower UGA suppression activity in vivo than the C deletion mutation at position 1054 in the 16S rRNA (12, 23).

None of the ISM586-positive clones were expressed on strain ISM668. This strain has been reported to act as a suppressor of UGA codons. However, it also displays other phenotypic alterations, such as a general increase in missense error frequency and suppression of all three nonsense codons, which may have interfered with the expression of the lambda clones.

Library screening on strain ISM586 allows the identification of clones which might never be detected with traditional methods due to premature truncation of cloned mycoplasmal proteins. However, library screening on this strain has some limitations. Induction of the opal suppressor tRNA in the presence of an altered RF-2 protein appears to have detrimental effects on cell growth and viability, which adversely affects the quality of the bacterial lawns. This problem can be overcome to a certain extent by improving the nutritional content of the medium and by increasing the number of cells that are used per plate in order to compensate for cell death.

Lambda growth also appears to be limited in strain ISM586 after induction of suppressor activity. Phage titers appear to be lower on this strain, plaques are tiny, and infected liquid cultures fail to lyse when grown under inducing conditions. This phenomenon can probably be accounted for by the effects of readthrough of UGA termination codons. Wada et al. (34) determined the frequency of codon usage in 65 genes from bacteriophage lambda. UGA was the most frequently used termination codon in these genes; 54% of the termination signals were encoded by UGA, whereas UAA and UAG accounted for 38% and 8%, respectively. Analysis of codon usage in 1187 *E. coli* genes revealed that UAA accounted for 67% of the termination codons in these sequences, whereas UGA and UAG represented 27% and 6.7%, respectively (34). If UGA codons are used to signal termination of phage or host proteins which are essential for phage growth, readthrough of these codons may generate proteins whose functional activity is impaired.

The restrictions that are imposed on this expression system due to poor phage growth can be partially remedied by using a nonsuppressor, recombination deficient strain to amplify each clone to a higher titer before infecting the suppressor strain. This can be accomplished by amplifying in microtiter plates or by using membranes lifted from one lawn to inoculate a second lawn. These additional steps make the library screening process slightly more cumbersome. However, they do result in the ability to detect sequences that would not normally be perceptible.

Further analysis of the lambda clones which were positive only on the suppressor strains is in progress. The two Lambda Zap II clones which were positive with the immune swine serum but not with the hyperimmune rabbit serum are of special interest. These clones appear to be identical by restriction enzyme analysis, and they probably contain the same genetic sequence. The differential response to the swine and rabbit antisera may simply reflect a difference in epitope recognition between species. However, the epitope(s) recognized may represent an epitope that is only recognized with swine immune serum and as such might be of interest as a potential vaccine candidate.

As we have demonstrated with the expression of the P1 adhesin (31), this system might also be beneficial after gene isolation for further analysis of gene products or for vaccine or diagnostic kit antigen production.

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Table 1. *E. coli* strains

Strains	Genotype	Reference
ISM586	YN2980 pISM3001	(31)
ISM663	JE105 pKKA1054	M. O'Connor
ISM666	MDA6650 pISM3001	This work
ISM667	ISM663 pKKA1054 pISM3001	This work
ISM668	rpsD12 pISM3001	This work
JM105	Δ[*lac-pro*] *thi strA supE endA sbcB15 hsdR4* {F' *traD36 proAB lacI^q lacZΔM15*}	Pharmacia
LE392	F$^-$ *hsdR514* (rk–, mk–) *lacY1* or Δ(*lacIZY*)6 *supE44 supF58 galK2 galT22 trpR55 metB1*	Stratagene
MDA6650	*rrsB* (SuUGA-ΔC1054) *glyV55* Δ(*tonB trpAB17*)/ F' *trpA*(UGA211)	(21)
P2392	LE392 (P2 lysogen)	Stratagene
rpsD12	S4 *ram* mutant	(2)
SMR10	lambda packaging strain	(27)
YN2980	*leu*(UGA) *lacZ659*(UGA) *his29*(UAG) *trpA9605*(UAG) *ilv thyA metB argH rpoB rpsL prfB3* Tn*10*	(15)
XL-1 Blue	*recA1 lac endA1 gyrA46 thi hsdR17 supE44 relA1* {F' *proAB*$^+$ *lacI^q lacZΔM15* Tn*10*}	Stratagene

Table 2. Reactivity of selected lambda recombinant clones using swine immune (S195) and rabbit hyperimmune (R322) antisera in different *E. coli* genetic backgrounds

	LE392		ISM586		ISM666	
Clone	R322	S195	R322	S195	R322	S195
1Z.1	-	-	+	+	±	±
1Z.2	-	-	+	+	±	±
1Z.3	-	-	+	+	±	±
1Z.4	-	-	-	+	-	-
1Z.5	-	-	+	+	±	±
1Z.6	-	-	-	+	-	-
1F.1	-	-	+	+	+	+
1F.2	-	-	+	+	+	+
1F.3	-	-	+	+	±	±
1F.4	-	-	+	+	±	±
1F.5	-	-	+	+	±	±
1F.6	-	-	+	+	±	±
1F.7	-	-	+	+	+	+
1F.8	-	-	+	+	+	+
1F.9	-	-	+	+	±	+
1F.12	-	-	+	+	+	±
1F.13	-	-	-	±	-	±
1F.14	-	-	-	±	-	±
1F.15	-	-	-	±	-	±
1F.16	-	-	+	+	+	+
1F.17	-	-	+	+	+	+
1F.18	-	-	-	±	-	±

GENERAL SUMMARY

Conclusions

The alternative codon usage pattern displayed by many mycoplasma species places limitations on the application of *E. coli* cloning hosts for the isolation of mycoplasma genes. The use of the UGA termination codon to encode tryptophan in mycoplasma sequences results in premature truncation of cloned proteins when expression in *E. coli* is attempted. Truncated proteins often escape detection during immunological screening or protein activity assays. We have developed a cloning system that enhances the expression of mycoplasma proteins by facilitating the insertion of tryptophan at UGA sites. The efficiency of an opal suppressor tRNA (*trpT*176) was augmented with other mutations [*prfB*3 or *rrsB*(SuUGA-ΔC1054)] which also display UGA suppression due to their influence on termination events.

We investigated the efficacy of the system by using it in the expression of well characterized mycoplasma sequences cloned into a prokaryotic expression plasmid. A fragment containing 4 TGA triplets and encoding a 72 kDa protein, which could not be expressed in the nonsuppressor strain JM105, was expressed in the presence of suppressor mutations. The addition of the *trpT*176 allele on a compatible plasmid allowed the read through of 3 UGA codons in the JM105 genetic background, although the level of expression was not high. The combination of the *prfB*3 mutation in strain YN2980 with the *trpT*176 allele appeared to enhance expression; three UGA codons were readthrough with a relativly high efficiency and the fourth was also suppressed to a lesser extent. Detectable expression could not be generated with the *prfB*3 mutation alone. When the *trpT*176 allele was combined with the *rrsB*(SuUGA-ΔC1054) mutation in strain MDA6650, similar results were obtained; strong readthrough of 3 UGA codons and some readthrough of the fourth codon was observed. Interestingly, this genetic background also generated additional products, which probably reflects translation reinitiation downtream of UGA sites or degradation of protein products.

After demonstrating suppression of four UGA codons, we also attempted expression of the gene encoding the P1 adhesin from *M. pneumoniae*. This gene encodes 21 UGA codons which hampered initial cloning attempts. The gene was ultimately cloned and sequenced by methods that did not require complete protein expression. We monitored expression using constructions with or without the signal sequence to eliminate the possibility that incomplete processing of the signal peptide or insertion into the membrane might hamper detection of protein products.

The construction containing the signal sequence generated a 40 kDa product in the presence of *trpT*176 in both the *prfB*3 and *rrsB*(SuUGA-ΔC1054) genetic backgrounds. Depending upon whether or not the signal sequence was cleaved, this indicates the readthrough of 3 to 6 UGA codons. Better results were obtained with the alternate construction lacking the signal sequence. The readthrough of 9-10 UGA codons was seen when *trpT*176 was combined with *prfB*3, and the suppression of 4-6 UGA codons was seen when *rrB*(SuUGA-ΔC1054) was used in conjuction with *trpT*176.

We have demonstrated that our expression system, consisting of combinations of opal suppressor mutations, can be used to enhance the expression in *E. coli* of mycoplasma sequences encoding UGA codons. The differences in expression efficiencies of the release factor 2 (*prfB*3) versus the 16S rRNA [*rrsB*(SuUGA-ΔC10540] mutations probably reflect the particular mechanisms of suppression in each case and the corresponding sensitivity and response to context effects. This system should find a variety of potential applications in the production of mycoplasma proteins in *E. coli* for further analysis or commercial applications.

An especially valuable application of an *E. coli* expression system for mycoplasma proteins is in the screening of genomic libraries. Suppression of UGA termination signals in mycoplasma sequences expands the usefulness of library analysis and provides for the isolation of genes that might never be detected using other methods. We employed strain ISM586 (*prfB*3/*trpT*176 mutations) in the expression of lambda libraries containing gene

sequences from *M. hyopneumoniae*. We demonstrated the expression of epitopes in suppressor strains which was not detected in the nonsuppressor strains which are commonly used for library screening.

Library screening with strain ISM586 does have certain limitations. The concurrent expression of *trpT*176 and *prfB*3 appears to have detrimental effects on cell growth and viability. In order to obtain lawns of an appropriate density, we grew large numbers of cells in rich media prior to induction of the *trpT*176 allele. However, the greatest liability lies in the fact that bacteriophage lambda growth appeared to be hampered by the suppression of UGA codons. Phage titers appeared to be lower on this strain, plaques were tiny, and infected liquid cultures failed to grow. Readthrough of UGA codons in essential phage or host protein genes probably explains impaired phage replication. We remedied poor phage growth during library screenings by amplifying each clone to a higher titer on a nonsuppressor strain prior to inoculation of the suppressor strain. Library screening with this intermediate step is slightly more cumbersome, but is useful in the detection of previously imperceptible gene products. Strain ISM666 displays better growth charateristics in conjuction with a weaker UGA suppresion activity. This strain is simpler to manipulate and may provide a sufficient level of suppression in some instances.

Future Studies

In this series of experiments, we investigated the usefulness of several opal suppressor mutations for the readthrough of UGA codons in mycoplasma sequences. Time constraints prevented us from fully exploring the potential of the expression system. There are a number of experiments that might be done in order to look at the efficiency of each combination of suppressor mutations in a particular context. A series of opal mutations in a *lacI-lacZ* fusion generated by Miller (53) could provide a test system for exploring the contribution of each genetic lesion to overall suppression efficiency, as well as the effect that context has on the

suppression process. Other cloned reporter genes could also be mutagenized in a similar manner to incorporate nonsense codons into coding sequences such that opal suppression is required for detectable functional activity.

No matter how many artificial contructions are analyzed, ultimately the true test of the system's usefulnes will come with it's employment in the expression of gene sequences of mycoplasma origin. There presently exist several mycoplasma sequences which have been cloned and sequenced and which have been found to encode multiple UGA codons. In most instances, reagents and techniques for the detection of full-length gene products are availabe or could be generated in the form of monoclonal or polyclonal antibodies or assays for gene activity. A partial list of these cloned sequences would include ORF6 of the P1 operon (7 UGAs) (85), the HMW3 adherence-associated gene (2 UGAs) (63), lambda gt11 clones containing fragments of *M. pneumoniae* DNA (6 and 3 UGAs)(79), the urease genes from *U. urealyticum* (8 UGAs) (7), the arginine deiminase genes from *M. arginini* (4 UGAs) (43) and *M. hominis* (5 UGAs) (24), the putative transposase in the RS-1 repetitive element from *M. hyorhinis* and *M. hyopneumoniae* (4 UGAs) (19), a portion of the ribosomal L16 protein from *M. capricolum* (3 UGAs) (97), the P29 (1 UGA), P37 (7 UGAs), and P69 (15 UGAs) putative transport system components from *M. hyorhinis* (16), and the capsid protein of the SpV4 spiroplasma virus (9 UGAs) (75).

When the results of our experiments with the P1 gene have been released, additional reports on the effectiveness of the system may come from a variety of sources as investigators use this system in the expression of their particular genes of interest. Suppression efficiencies may be even greater than we observed, depending upon the distribution of the UGA codons within a particular gene and the context surrounding each codon.

The usefulnes of our system could be enhanced if the *prfB*3 or the [*rrsB*(SuUGA-ΔC10540)] mutations were made inducible like the *trpT*176 allele. Cloning and cell growth could be carried out under noninducing conditions, and the detrimental effects of all opal

suppressor mutations could be prevented until the time of screening. The *prfB*3 mutant allele has been cloned into the *Eco*RI site of plasmid pACYC184 to generate plasmid pNE6 (52). The *prfB*3 allele could subseqently be subcloned behind an inducible promoter. A wild type copy of the *prfB* gene would have to be maintained in the cell to provide release factor activity before induction of the *prfB*3 allele. Wild type release factor is usually present at limiting concentrations within the cell, and overexpression of the mutant allele behind a strong promoter from a multicopy plasmid should compete effectively with the wild type protein after induction. The UGA suppression activity displayed by the mutant release factor should also lead to the production of nonfunctional protein from the wild type allele. Translational regulation of this allele occurs through a one base pair frameshift and subsequent avoidance of an in-frame internal UGA codon. Enhanced binding of the suppressor tRNA might interfere with both termination and the frameshift and prevent expression of functional release factor-2 protein due to translation into a nonproductive reading frame.

The *rrsB* operon containing the C1054 deletion has been cloned behind the P_L promoter to generate pNOC1054 (68) which renders it inducible at 42°C in the presence of the C_{I857} lambda repressor. This might be cloned behind other inducible promoters as well. This mutation is dominant to the other ribosomal operons within the *E. coli* genome (56).

The expression system could also be enhanced through the development of mobile opal suppressor mutations that could be transfered to any *E. coli* genetic background which contained a desirable genotype for a particular application, such as a host lacking restriction, modification, or recombination activites, or a host containing a genetic lesion in the chromosome for the purpose of cloning by complementation. The *prfB*3 allele can be transfered via P1 transduction with selection for a closely linked Tn*10* insertion (38). Since the opal suppressor mutations *trpT*176, *prfB*3, and *rrsB(SuUGA- ΔC10540)* have been cloned (22, 52, 56, 71), these genes could be subcloned into vectors with the appropriate antibiotic resistance and origin of replication to make them compatible with other cloning vectors. Two

opal suppressor alleles might be cloned into the same episome to expedite transfer to another host. Such a plasmid might have potential application in mini cell systems for expression of specific cloned fragments of mycoplasma origin.

In addition to all of the modifications which could be made to the system consisting of the *trpT*176, *prfB*3 or *rrsB*(*SuUGA- 1054*) mutations, other mutations which exhibit opal suppressor activity could also be obtained and combined for the expression of mycoplasma sequences.

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