Construction of *Escherichia coli*-*Porphyromonas gingivalis* conjugal shuttle vectors

by

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Introduction

Periodontal diseases are a group of infectious diseases affecting the alveolar bone, cementum, gingiva, and periodontal ligament supporting the teeth. Periodontitis results in the progressive loss of these supporting structures, and ultimately the loss of teeth. The prevalence of periodontal disease in the population is relatively high. For example, a study of over 15,000 adults from 18-64 years of age revealed mild periodontitis occurred in 44% of the sample population, while moderate periodontitis was found in 13%.[6] The American Academy of Periodontology has concluded that 5-20% of the U.S. population suffer from severe, generalized periodontitis, while a majority of adults suffer from mild-to-moderate periodontitis.[7]

The primary etiology of periodontal disease is bacterial plaque. Numerous studies have shown that removal of existing plaque and prevention of further plaque accumulation results in a reduction in clinical manifestations of periodontitis. The composition of dental plaque is diverse, and marked differences have been observed between the subgingival flora of healthy and periodontally diseased patients. Certain anaerobic flora, including *Porphyromonas gingivalis*, *Eubacterium* species, *Peptostreptococcus micros*, and spirochetes, are present in increased proportions in periodontitis patients.[61,62] These so-called putative periodontopathogens are implicated in the destruction of periodontal tissues.

Role of *Porphyromonas gingivalis* in periodontal disease

Porphyromonas gingivalis is an asacchrolytic black-pigmented bacterium widely implicated in the pathogenesis of periodontal diseases.[60] Black-pigmented oral bacteria, particularly *Porphyromonas* and *Prevotella* species (both formerly in the genus

Bacteroides), have long been associated with the presence of periodontitis.[17, 93, 103, 108, 116, 124] For example, a survey of 138 patients with untreated severe periodontitis reported that *P. gingivalis* was isolated in 48% of test sites.[82] A comparison of site prevalence of *P. gingivalis* in healthy, elderly, and periodontitis patients revealed 4, 5, and 23% positive sites, respectively, as well as an odds ratio of 17.9:1 for periodontitis in patients harboring *P. gingivalis* in four or more sites.[26] Similarly, other studies have found a high occurrence (> 40%) of *P. gingivalis* in destructive adult periodontitis lesions.[54, 92, 97] Tanner and colleagues evaluated the subgingival flora, by culture and darkfield microscopy, in progressive periodontal disease and reported higher proportions of *P. gingivalis* in sites losing alveolar bone than in stable sites.[108] Dzink and coworkers found elevated levels of *P. gingivalis* among the predominant cultivable flora of destructive periodontitis lesions, as well as an increased likelihood of disease activity in the presence of *P. gingivalis*.[16] Furthermore, reduction of *P. gingivalis* in periodontitis patients correlates positively with both a reduction in probing depth and a gain in clinical attachment.[81]

P. gingivalis has also been associated with less common forms of periodontal disease such as refractory periodontitis, periodontitis associated with systemic disease, and early-onset periodontitis. In a study of treated patients, Haffajee and coworkers showed elevated mean levels of *P. gingivalis* in refractory patients and patients with recurrent disease.[28] Based on culture and immunofluorescence microscopy of subgingival plaque, Zambon and coworkers implicated *P. gingivalis* as a prominent feature of moderate to severe periodontitis in Type II diabetes patients.[124] In a study of non-localized juvenile periodontitis, *P. gingivalis* was isolated from 5 of 6 cases with

moderate periodontal breakdown; cases with minimal periodontal breakdown did not harbor *P. gingivalis* in either the deepest periodontal pocket or the tongue.[113] Similar results were found in a study by Moore and coworkers, where *P. gingivalis* was identified in the subgingival flora of juvenile periodontitis patients.[62]

P. gingivalis is able to elicit a humoral immune response in periodontitis patients.[63] Juvenile periodontitis patients demonstrate elevated serum antibodies to *P. gingivalis*.[10, 109] A comparison of antibody responses to *P. gingivalis* in adult and early-onset periodontitis revealed that 47% of adult periodontitis patients, 36% of early-onset periodontitis patients, and 33% of localized juvenile periodontitis patients were seropositive compared to 5% of healthy controls.[9] Review of the antibody subclasses suggests that IgG_3 may confer protection against periodontal diseases, including localized juvenile periodontitis, whereas IgG_1 and IgG_4 are elevated in patients positive for *P. gingivalis* and periodontal disease.[74]

The association between *P. gingivalis* and periodontitis has also been demonstrated in animal models. Holt and colleagues reported development of destructive periodontal lesions after local infection of non-human primates with *P. gingivalis*.[34] Roeterink and coworkers injected suspensions of *P. gingivalis* into the palates of rats and evaluated the effects histologically. They noted an influx of neutrophils within the first 2 to 4 hours, followed by a mononuclear infiltrate. The periosteum was initially lost, with evidence of bone resorption starting after 8 hours and continuing over 10 days.[83]

In human studies, reduction or elimination of *P. gingivalis* has resulted in a reduction of periodontal destruction. Zack and coworkers reported a reduction in clinical manifestations of periodontitis correlated with reduction of *P. gingivalis* antigens as a

result of periodontal therapy.[122] Reduction or elimination of *P. gingivalis,* either by administration of antibiotics or conventional periodontal therapy, results in resolution of clinical signs of inflammation.[27, 49, 81, 94, 96, 99, 102] *P. gingivalis* was designated by Socransky and colleagues as a component of the "red complex," (along with *Bacteroides forsythus* and *Treponema denticola*, which has been associated with increases in probing depths and bleeding on probing.[100] Due to the evidence linking *P. gingivalis* to periodontitis, the 1996 World Workshop in Periodontics designated *P. gingivalis* as an etiologic agent of adult onset periodontitis.[123]

Mechanisms of periodontal destruction

P. gingivalis is thought to induce periodontal destruction through production of various products. *P. gingivalis* produces outer membrane vesicles and blebs which contain virulence factors such as lipopolysaccharide (LPS), collagenase, and other proteases. The lipopolysaccharide (LPS) produced by *P. gingivalis* has been shown to stimulate bone resorption, inhibit gingival fibroblast proliferation, and inactivate complement proteins. Takemura and colleagues showed that *P. gingivalis* LPS can increase periodontal ligament cell proliferation and responsiveness to platelet-derived growth factor (PDGF).[107] Both LPS and proteases obtained from sonicated extracts of *P. gingivalis* have been shown to inhibit osteogenesis in a osteoblast cell culture.[55] In addition, *P. gingivalis* LPS stimulates differentiation of osteoclasts, release of prostaglandin E_2 (PGE_2), tumor necrosis factor alpha (TNF-alpha), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-1ß (IL-1ß), inflammatory cytokines associated with destruction of periodontal tissues, from human gingival fibroblasts, neutrophils and macrophages *in vitro*.[1, 5, 11, 44, 91, 121] LPS also stimulates release of IL-1 receptor

antagonist, which inhibits IL-1ß.[121] Biological activity of LPS may vary between strains of *P. gingivalis*.[70, 87]

P. gingivalis proteases

One of the more widely studied virulence factors of *P. gingivalis* is protease activity. Because *P. gingivalis* is an asacchrolytic bacterium, it metabolizes proteins for energy. *P. gingivalis* is characterized by "trypsin-like" protease activity. This is actually the result of three major proteases (RgpA, RgpB, and Kgp) specific for either lysine or arginine.[50,77, 78, 80] Additional proteases, including Tpr and PrtT have also been isolated.[4, 72] Protease-deficient mutants have demonstrated lower growth rates[73, 111, 114] reduction or absence of hemagglutination,[37, 88] reduced virulence in mice,[21] and reduction or absence of fimbriae.[110, 111] Tokuda and colleagues noted that mutants of *P. gingivalis* W83 deficient in protease arg-gingipain B express a decreased number of fimbriae, while mutants deficient in arg-gingipain A are not able to express fimbriae.[110, 111] These mutants show a decreased expression of the *fim*A gene, which suggests that the proteases have a regulatory function in cell growth and development.

Modulation of host response by _P. gingivalis_

Once established within the gingival crevice, proteases may enable *P. gingivalis* to evade the host response. Kilian demonstrated that *P. gingivalis* proteases degrade immunoglobulins IgA1, IgA2, and IgG.[48] Sundqvist and coworkers showed that proteases from black-pigmented *Bacteroides* are able to degrade IgG, IgM, C3, and C5.[105] These results are in contrast with those of Nilsson and colleagues, who did not report any degradation of immunoglobulins by *P. gingivalis* W83.[67] In addition, Jagels and coworkers demonstrated breakdown of the C5a receptor on human neutrophils by

two vesicle-bound proteases (serine protease and lys-gingipain) of *P. gingivalis*.[45] *P. gingivalis* has also been shown to inactivate C1 inhibitor, antithrombin, plasminogen, prekallikrein, prothrombinase complex, clotting factor X, and break down fibrinogen.[52] The suspected mechanism for this activity was that the proteases acted directly on the host molecules.[51] Grenier and coworkers implicated lipopolysaccharides and proteases present in outer membrane vesicles of *P. gingivalis* in the inactivation of human serum bactericidal activity.[24, 25] They also isolated an 80-kDa protease from *P. gingivalis* which inhibited serum bactericidal activity against *Capnocytophaga ochracea.*

Another possible mechanism of host evasion is decreased opsonization. Scheinkein and colleagues developed a strain of *P. gingivalis* defective for the *prt*H gene.[85] These mutants were less able to break down complement C3, and greater numbers of C3 opsonins were observed on their cell surfaces. In a mouse model, these mutants were less virulent than their parent strain. The net result of these effects on the immune system is that the host is unable to kill unopsonized bacteria. In addition, the degradation of antibodies and other immune system components may allow other pathogenic bacteria to infect the periodontal pocket.

P. gingivalis proteases have been shown to affect extracellular mediators of inflammation. Gingival epithelial cells normally secrete IL-8, a chemoattractant for neutrophils, in response to pathogenic oral flora. When these cells are incubated in the presence of *P. gingivalis*, however, IL-8 accumulation is reduced; this phenomenon is observed even when other species of plaque bacteria are incubated along with *P. gingivalis*.[12] These results suggest that *P. gingivalis* inhibits neutrophil migration, which is a first step in the host response to periodontopathic bacteria. This is consistent with the

observation of Madianos and colleagues, who observed that *P. gingivalis* infection of epithelial cells in culture inhibited transmigration of neutrophils across a membrane barrier.[56] Moreover, *P. gingivalis* inhibited FMLP-, interleukin-8, or pathogenic *E. coli*-stimulated neutrophil migration. *P. gingivalis* has also been shown to degrade tumor necrosis factor alpha, a cytokine which modulates the host inflammatory response.[8] Fletcher and coworkers found that extracts of *P. gingivalis* were able to break down cytokines IL-1ß, IL-6, and the IL-1 receptor agonist.[22] Additionally, exposure of IL-1ß to *P. gingivalis* caused IL-1ß to lose its biological activity. Similarly, Huang and coworkers showed *P. gingivalis* protease-mediated breakdown of IL-8 and intercellular adhesion molecule-1, suggesting that these proteases are able to modulate the neutrophil response.[39] *P. gingivalis* proteases also have potent effects on neutrophil toxicity.[45] Odell and coworkers reported that *P. gingivalis* was able to inactive non-oxidative killing mechanisms of neutrophils such, as cathepsin G, elastase, bacterial-permeability increasing factor, and defensins.[68]

P. gingivalis proteases also have effects on the host vascular system. Nilsson initially reported alteration of the serum proteinase cascade, including clotting factors, in the presence of *P. gingivalis*.[67] Imamura and coworkers[41] described alterations in Factor X as a result of *P. gingivalis* proteases, confirming Nilsson's report. In addition, *P. gingivalis* proteases have been shown to degrade serum fibrinogen, an important factor in early clot formation.[86] These effects are partly responsible for the characteristic bleeding on probing noted in periodontitis patients. *P. gingivalis* proteases also affect the kallikrein/kinin cascade.[32, 40, 67] Hinode and colleagues reported that the activity of a specific protease (protease C) from *P. gingivalis* strain 381 was primarily responsible for

kallikrein activity, which results in the release of bradykinin.[32] Bradykinin causes vascular dilation and increased permeability, which in turn provides a source of nutrients for the subgingival plaque flora.

Destructive effects of *P. gingivalis* proteases

P. gingivalis proteases have been shown to have both direct and indirect destructive effects on periodontal tissues. Type I collagen is readily degraded by *P. gingivalis*; both proteases [3, 46] and LPS[98] have been implicated in this activity. In addition, *P. gingivalis* proteases may degrade extracellular matrix components. In *in vitro* studies, *P. gingivalis* has been shown to bind and denature fibronectin and other extracellular matrix molecules.[51, 52] Both trypsin-like proteases and lipopolysaccharide have been implicated in this activity.[98] Uitto and coworkers incubated human gingival fibroblasts with extracts obtained from *P. gingivalis*. They noted degradation of fibronectin and cell surface glycoproteins by whole extracts as well as a purified *P. gingivalis* protease.[112] Type IV collagen, which is predominant in epithelial basement membranes, is also degraded by *P. gingivalis* proteases.[46]

Indirect effects on the periodontium mediated by *P. gingivalis* proteases include induction of host matrix metalloproteinases and degradation of plasma proteinase inhibitors. Sorsa and colleagues found that extracts of *P. gingivalis* were able to activate procollagenases MMP-1 and MMP-8 from fibroblasts without directly affecting collagen or tissue inhibitor of metalloproteases (TIMP-1).[101] Once the collagen is cleaved by host collagenases, *P. gingivalis* is able to degrade the fragments through protease activity. *P. gingivalis* directly induces degranulation of PMNs and activation of metalloproteases MMP-1 and MMP-8.[12, 15, 101] In addition, it can activate precursors of host proteases by

enhancing plasminogen activator secretion, which in turn leads to activation of host procollagenases. Uitto and colleagues observed induction of plasminogen activator and host collagenases in human fibroblasts cultured in the presence of *P. gingivalis* extracts, suggesting that collagenase activity in periodontal disease may be host cell mediated.

In addition to destruction of periodontal tissues, *P. gingivalis* proteases may also prevent repair and regeneration.[112] Matsuda and coworkers reported a reduction in both mitogenic and chemotactic effects of platelet-derived growth factor (PDGF) in the presence of *P. gingivalis* extracts. In human periodontal ligament cell culture models, purified *P. gingivalis* proteases appear to stimulate epithelial cells and fibroblasts to upregulate collagenase and stromelysin production, resulting in degradation of collagen matrix and accumulation of procollagenase.[59] These findings imply that the proteases isolated from *P. gingivalis* can induce phenotypic changes in host cells.

Colonization of the oral cavity by *P. gingivalis*

Attachment to mucosal surfaces is necessary for colonization of the oral environment. However, *P. gingivalis* does not appear to colonize mucosal surfaces directly, but attaches to Gram-positive early colonizing flora.[60] This attachment is often facilitated by fimbriae.[60] Expression of fimbriae appears to be regulated in part by proteases, since protease-deficient *P. gingivalis* mutants show decreased binding to Gram positive oral flora.[53, 110] Fibronectin, an adhesion molecule of the host extracellular matrix, is able to inhibit adhesion of *P. gingivalis* to cell surfaces by binding to the fimbrillin component of fimbriae.[64] In animal models, fimbriae are able to stimulate host response, including T-cell activation and IgG production.[42, 69] Fimbriae also stimulate bone resorption *in vitro* by inducing expression of IL-1ß and granulocyte macrophage

colony-stimulating factor, which in turn induce differentiation of osteoclasts.[31, 47] Malek and coworkers reported a fimbriae-deficient mutant of *P. gingivalis* 381 was inhibited both in its ability to coaggregate with *Streptococcus gordonii* and to induce periodontal bone loss in a gnotobiotic rat model in comparison to the wild type.[57]

Hemagglutination by *P. gingivalis*

P. gingivalis also demonstrates hemagglutinating activity.[60, 95] Okuda and Takazoe demonstrated hemagglutination in 29 of 59 strains of black-pigmented *Bacteroides*, including *P. gingivalis*.[71] It has been suggested that hemagglutinins may facilitate bacterial penetration into host cells *in vitro*, however, this has not been conclusively demonstrated *in vivo*.[18] Hemagglutination is most likely a multifactorial phenomenon. Okuda and Takazoe suggested that hemagglutination was mediated by fimbriae, based on an observation that only fimbriated strains had hemagglutinating activity.[71] However, *P. gingivalis* mutants deficient for the *fim*A gene have demonstrated hemagglutination.[29] Moreover, monoclonal antibodies directed against fimbriae inhibited adherence of *P. gingivalis* to cell surfaces, but did not affect hemagglutination.[43] These observations suggest that fimbriae are not the sole determinant of hemagglutination. The observation that pigment-deficient mutants of *P. gingivalis* also have decreased hemagglutination further supports the theory that hemagglutination depends on a number of factors.[38] Several studies have shown protease-deficient *P. gingivalis* mutants to have reduced hemagglutinin activity compared to wild-type.[37, 38, 65, 88, 111, 117] Pike and colleagues studied the adhesins of *P. gingivalis* and concluded that they are complexes of proteases and adhesion proteins, including hemagglutinins.[75, 76] Further study of these complexes showed that a decrease in fibronectin, fibrinogen, or laminin in media resulted

in a decrease in hemagglutination. These results imply that hemagglutination is a laboratory correlate of *P. gingivalis* adhesin complexes rather than a dedicated pathologic mechanism.

The multifactorial nature of hemagglutination may be explained by genetic analysis. Han and colleagues described the *hag*A gene in *P. gingivalis*, which codes for a putative hemagglutinin.[30] The *hag*A protein has domains homologous to those encoded by *rgp*A and *kgp* genes, suggesting that gene rearrangements may be responsible for separate hemagglutinin and protease genes and may explain the decreased hemagglutination associated with protease-deficient mutants. Further evidence of the interaction between proteases and hemagglutination has been elicited through genetic studies. Sequence homologies have been shown between *rgp*A, *rgp*B, *kgp*, and *hag*A genes, as well as decreased hemagglutination in protease-deficient mutants. Shi and colleagues demonstrated that hagA has an adhesion domain region at the C-terminals of both rgpA and kgp proteases.[88] Triple mutants for these three genes show no hemagglutination.

Molecular biology of *P. gingivalis*

In order to study these virulence factors and their complex interactions, and to clarify their functions in the pathogenesis of periodontitis, researchers must apply techniques of molecular genetics. These include cloning, gene inactivation, and complementation. One example of how such techniques are used is the study of the *fimA* gene in *P. gingivalis*. The gene was initially isolated and cloned from chromosomal DNA into a plasmid (pUC13Bg12.1).[14] Although the gene was shown to encode fimbrillin, there was no direct genetic evidence that *fim*A was required for fimbrial

expression. Hamada and coworkers constructed a mutant deficient for *fim*A by using a "suicide vector:" an extrachromosomal DNA fragment intended to enter the target cell and integrate into the chromosomal DNA.[29] This vector contained the mutant *fim*A gene as well as antibiotic resistance sequences to allow growth of transconjugants on selective media. The resulting mutants were then grown and characterized. This approach has also been utilized in studying several protease gene products, including arg-gingipain,[66] *prt*T,[72] *Tpr*,[73] and *prt*H.[21]

The *prt*H gene encodes a 97-kDa protease capable of hydrolyzing complement component C3.[20] However, disruption of the *prt*H gene produced a mutant deficient in both the encoded 97-kDa protease as well as a 45-kDa protease. This presents a dilemma in that reduction of virulence in this mutant could be attributed to either or both proteases. Furthermore, it is unclear whether the 45-kDa protease is activated by the 97-kDa protease, derived from a common precursor of the 97-kDa protease, or if its expression is controlled by a promoter that is affected by the disruption of the *prt*H gene. This uncertainty may be resolved by using genetic complementation. In this technique, a wild-type gene is cloned into a plasmid which is then introduced into the mutant strain. The plasmid remains extrachromosomal, so the gene product is restored without restoring effects of transcriptional polarity such as operons. This enables the investigator to determine the function of the gene as well as its effects on accessory genes and transcriptional polarity. Figure 1 provides a schematic representation of complementation. In the *prt*H example, if re-introduction of the gene by complementation yielded both proteases, then the 97-kDa protease is responsible for production or regulation of the 45-kDa protease; if the 45-kDa protease is still absent,

then it is likely to be encoded in a transcriptional operon downstream of the *prt*H gene. Additionally, if only the 97-kDa protease is expressed, then its effects in an animal model or human cell culture can be studied directly.

Complementation is a powerful technique for elucidating gene function and interaction. However, attempts to develop shuttle vectors in *P. gingivalis* have been limited by a number of factors. Naturally-occurring plasmids have not been isolated from *P. gingivalis*.[33, 84, 120] Replicons from other genera do not function in transformation of *P. gingivalis*.[118] Furthermore, *P. gingivalis* has restriction enzymes which degrade incoming double-stranded DNA.

In spite of these challenges, attempts have been made to transfer genetic material into *P. gingivalis*. Progulske-Fox and colleagues used a conjugal *Bacteroides* plasmid shuttle vector, pE5-2, to transfer antibiotic resistance from *Escherichia coli* to *P. gingivalis* at a frequency of 1.4-2.0 x 10^{-7} transconjugants per recipient.[79] Plasmid R751::*Ω4 has been used as a suicide vector to deliver transposon Tn4351, which has been used to transfer erythromycin resistance genes into the *P. gingivalis* chromosomal DNA and induce pigment-deficient mutants.[35, 38, 89] Maley and coworkers successfully transferred a vector, pNJR-12, from *E. coli* to *P. gingivalis* at a rate of 4.5 x 10^{-4} transconjugants per recipient, which represents a substantial improvement over pE5-2.[58] The pNJR-12 vector system was later used to complement the *tpr* protease genes.[72] However, these pNJR-12 transconjugants were not stable and could not maintain growth on selective media.

Proposed research

A plasmid, pYHBA1, derived from *Porphyromonas asacchrolytica* has been used to develop a shuttle vector that is introduced into *P. gingivalis* by electroporation.[119] This shuttle vector is able to replicate stably, presumably due to the presence of the pYHBA1 plasmid. However, use of this system requires disabling of the *P. gingivalis* host restriction systems by nitrosoguanidine mutagenesis. Consequently, it is likely that secondary mutations occur within the *P. gingivalis* chromosomal DNA. These mutations may ultimately confound the results of complementation assays.

The objective of this project was to construct a shuttle vector that will transfer efficiently from *E. coli* to *P. gingivalis* and replicate stably within *P. gingivalis*. This objective is based on the hypothesis that the addition of *P. asacchrolytica* plasmid pYHBA1[36, 120] to existing vector systems will enhance its stability in *P. gingivalis*. Ultimately, the vector system should be able to accept subcloned genes for complementation experiments, and would serve as a valuable tool for future molecular genetic studies of *P. gingivalis*.

Materials and methods

Selection of vector components

The first construct attempted was designed to utilize five components:

1. pYHBA1, isolated from *P. asacchrolytica*,[36, 120] which is intended to allow stable replication in *P. gingivalis*.

2. An origin of replication from pBluescript (Stratagene, Inc.) that will function in *E. coli*.

3. An antibiotic resistance gene carried on pBluescript (Stratagene, Inc.) that will function in *E. coli* to allow selection of plasmid-carrying cells.

4. An erythromycin resistance gene, *erm*F, originally isolated from the *Bacteroides fragilis* plasmid pBF4.[104, 115] This will allow selection of transconjugants in *P. gingivalis*.

5. An origin of transfer, RP4 *ori*T, from plasmid RP4,[2] which will allow mobilization of the plasmid out of *E. coli*.

These fragments were obtained as follows:

A 5 ml culture of *P. asacchrolytica* ATCC 27067 was grown anaerobically at 37° C for 48-72 hours in supplemented trypticase soy broth (Appendix A). The bacterial cells were harvested by centrifugation and a "mini-prep" SDS-lysis plasmid preparation[23] was performed (Appendix B). The resulting plasmid DNA was digested with *Bam*H1 for one hour and analyzed by gel electrophoresis (for protocol Appendix B). Upon confirmation of a single 5 kb band consistent with pYHBA1 (Figure 2), the process of growing and harvesting cells was repeated with a 500 ml broth, and an SDS-lysis "maxi-prep" procedure (Appendix B) was performed. The plasmid DNA was further purified

by CsCl density gradient centrifugation and quantified by spectrophotometry (Appendix B). The resulting plasmid DNA was again verified by digestion with *Bam*H1 and agarose gel electrophoresis.

Preparation of initial construct

After verification of the purified pYHBA1 DNA, a chimeric plasmid was constructed utilizing the *Bam*H1 sites of pBluescript and pYHBA1. Each plasmid was digested with *Bam*H1, precipitated with ammonium acetate and isopropanol, washed with 70% ethanol, and redissolved in TRIS-EDTA (TE) buffer (Appendix B). These fragments were then incubated with T4 DNA ligase at 16° C (Appendix B).

To isolate the chimeric plasmid construct, the ligation products were precipitated and washed twice with distilled water, then added to 40µl of chilled electrocompetent *E. coli* XL-1 Blue cells and electroporated[19] (Appendix B). The electroporated cells were then suspended in 1 ml of SOC broth (Appendix A) and incubated at 37°C for one hour. After incubation, 0.1 ml portions of ten-fold serial dilutions of the broth were plated on L-XICT agar (Appendix A). This medium contains carbenicillin, X-Gal, and IPTG, which allows both antibiotic resistance selection and blue-white color screening. Only cells containing the pBluescript plasmid will be able to grow in the presence of carbenicillin. In addition, cells which contain pBluescript with pYHBA1 ligated into the *Bam*H1 site will produce white colonies; cells lacking this component will appear blue.

To confirm the presence of the chimeric plasmid, white transformant colonies were selected and restreaked onto L-XICT plates, incubated overnight at 37°C, then grown overnight in 5 ml SOB (Appendix A) broth supplemented with carbenicillin and tetracycline. Plasmid DNA was extracted from the cells by the miniprep procedure and

evaluated by agarose gel electrophoresis. The construct was confirmed by the presence of 3 kb and 5 kb fragments in the gel (Figure 8). A plasmid maxiprep was then performed, and CsCl density gradient centrifugation was used to purify a sufficient quantity of plasmid for further experiments. The chimeric plasmid, named pDC1-12 was then mapped by restriction with *Bam*HI, *Sac*I, *Cla*I, and *Bgl*II (Figure 3).

XL-1 Blue *E. coli* cells containing pDC1-12 were grown in broth and plasmid DNA extracted by the maxiprep procedure. The plasmid was then digested with both *Pst*I and *Eco*RI. The resulting 8 kb fragment was separated by gel electrophoresis and extracted from the gel using the QIAEX system (Qiagen, Valencia, CA).

The erythromycin resistance and pBlue *ori*T sequences were harvested using similar techniques. *E. coli* cells containing the plasmid pBlue 3.8-10 (Hoover, unpublished construct) were grown in SOB broth as previously described. Plasmid DNA was extracted by the miniprep procedure. To isolate the *erm*F erythromycin resistance gene, the plasmid was then digested with and *Cla*I and separated by gel electrophoresis. The resulting 2.8 kb fragment containing the *erm*F gene was excised from the gel and extracted using the QIAEX system.

Purified pBlue *ori*T plasmid DNA (Hoover, unpublished construct), which contains the *ori*T cassette from pRP4[2], was digested with *Pst*I and *Cla*I. The fragments were separated by gel electrophoresis, yielding fragments of approximately 0.4 and 3 kb (Figure 9). The 0.4 kb fragment was extracted from the gel by the QIAEX system.

With the three plasmid fragments isolated, a three-part ligation was attempted, based on the restriction sites of each fragment. The *ori*T fragment had been restricted by *Pst*I and *Cla*I, with the intent of ligating the *Cla*I terminus to the *Cla*I terminus of the

ermF fragment. The *Pst*I terminus of the *ori*T fragment was intended to ligate to the *Pst*I terminus of pDC1-12, while the terminus of the ermF fragment would ligate to the terminus of pDC1-12 (Figure 4).

The fragments were ligated with T4 DNA ligase per protocol (Appendix B), electroporated into electrocompetent *E. coli* XL1-Blue cells and plated on L-XICT agar. Transformant colonies, identified by their white color, were streaked, cultured and analyzed by miniprep extraction of DNA, restriction digests, and gel electrophoresis.

Preparation of second construct

A second construct was prepared utilizing the chimeric pDC1-12 plasmid, the *ori*T fragment from plasmid RP4, and erythromycin resistance from pBlue 5.8-10 (Hoover, unpublished construct) as follows. Purified pBlue *ori*T plasmid DNA, which contains the *ori*T cassette from pRP4[2], was digested with *Kpn*I and *Bam*HI as previously described. In addition, purified pBlue 5.8 was digested with *Sal*I and *Bam*HI to yield a 2.8 kb fragment. pYHBAI was digested with *Kpn*I and *Sal*I. The three digests were then separated by gel electrophoresis, extracted from the gel per QIAEX protocol, and ligated with T4 DNA ligase as previously described. The ligation mixtures were electroporated into electrocompetent XL-1 Blue *E. coli* cells. These cells were then plated on L-XICT. Eight transformants were selected following electroporation. These cells were restreaked and grown in SOB broth, and plasmid DNA was extracted by the miniprep procedure. Gel electrophoresis was performed to confirm presence of all components within the plasmid. DNA from a specific transformant (DC4-3) exhibited restriction characteristics consistent with the construct design. This plasmid was named pECPG3 (Figure 5). The ECPG3-containing cells were grown in broth, and a maxiprep procedure was performed

to extract larger quantities of DNA. Following confirmation of the maxiprep products by gel electrophoresis, pECPG3 was electroporated into S17 *E. coli* cells. In addition, pECPG3 was electroporated into *E. coli* strain XL1-Blue containing plasmid R751, which contains a trimethoprim resistance gene as well as genes allowing mobilization of plasmid vectors.

Preparation of third construct

Plasmid pNJR-12 (Figure 6) was used to attempt construction of a third vector. pNJR-12 was grown in *E. coli* and extracted by maxiprep procedure. The presence of pNJR-12 was confirmed by gel electrophoresis. The plasmid was then restricted with *Sac*I and separated by gel electrophoresis. The 4.5 kb pB8-51 fragment was discarded, and the remaining 10 kb and 2.5 kb fragments were ligated with T4 ligase. The ligation mixture was electroporated into XL-1 Blue cells, which were then plated on L-Kan plates (Appendix A). A single transformant was identified and grown in 5 ml broth. The plasmid DNA was then extracted by miniprep. Restriction with *Bam*H1 and gel electrophoresis revealed a 12.5 kb band, which is consistent with the sum of the two ligated fragments. This band was excised from the gel and the DNA extracted by QIAEX protocol. Previously purified pYHBA1 was restricted with *Bam*H1, and the two fragments were ligated with T4 ligase (Figure 7). The resulting ligation mixture was electroporated into XL-1 Blue cells and plated. A total of 42 colonies were streaked and then grown in 5 ml broths for miniprep procedures. Miniprep products were analyzed by gel electrophoresis.

Matings

Aerobic/anaerobic matings were conducted using several protocols (Appendix C). After mating, the spots were suspended in broth, diluted, and plated on selective media to determine transfer of the plasmid. In order to test all aspects of the mating protocol, several donor and recipient strains were used as follows:

1. pECPG3 x *P. gingivalis* 33277. The plasmid pECPG3 was electroporated into both S17 and R751-containing XL1-Blue *E. coli* cells and mated (Appendix C) with *P. gingivalis* 33277. This mating was carried out to determine whether the plasmid construct could be successfully mobilized out of *E. coli* and maintained in *P. gingivalis.*

2. *E.coli-E. coli* matings. pECPG3 was electroporated into *E. coli* strain S17 and strain XL1-Blue containing R751 as the donor strains and *E. coli* RVN as the recipient strain. These matings were intended to evaluate the mobilization of pECPG3 from the donor strain.

3. Matings utilizing known vector systems. *E. coli* strains containing the plasmid R751::Ω4[89] and the plasmid shuttle vector pNJR-12[58] were mated with *P. gingivalis* 33277. These matings were intended to determine the effectiveness of the mating protocol in introducing genetic material into *P. gingivalis* 33277.

Ampicillin resistance

In order to increase the selective pressure on the donor cells to retain the chimeric plasmid pECPG3 during matings, attempts were made to induce ampicillin resistance *P. gingivalis.* This would allow matings in the presence of ampicillin. Three stocks of *P. gingivalis* ATCC 33277 were incubated for 72 hours at 37° C in trypticase soy broth (TSB) supplemented with hemin, menadione, DTT, and ampicillin at concentrations of

0.1, 0.25, 0.5, 1.0 and 2.0 µg/ml (Appendix A). Cultures exhibiting growth were used to inoculate broths at higher concentrations and streaked onto LRBB plates containing 5µg/ml ampicillin. *P. gingivalis* was confirmed by darkfield microscopy and culture.

Results

Chimeric plasmid

The pYHBA1-pBluescript chimeric plasmid (pDC1-12) was successfully constructed by ligation of the 5 kb pYHBA1 and 3 kb pBluescript fragment. These results were confirmed by gel electrophoresis (Figure 8). Based on the components of the initial plasmid and digests with *Bam*HI, *Sac*I, *Cla*I, and *Bgl*II), a restriction map was created (Figure 2).

Initial construct

The first construct was attempted as described. A total of 31 white transformant colonies were selected, restreaked on L-XICT plates, grown in 5 ml SOC broth (Appendix A), and evaluated by miniprep procedure. However, digestion with *Eco*RI failed to demonstrate a 0.4 kb band indicative of the *ori*T segment, suggesting that the white colonies isolated from the electroporation mixture were false positives (Figure 10).

Second construct

The second construct, pECPG3, was successfully prepared and electroporated into both S17 *E. coli* cells and XL1-Blue/R751 *E. coli* cells. Gel electrophoresis of plasmid DNA extracted from the transformants confirms that the appropriate-size fragments are present (Figure 11). The 0.4 kb *ori*T fragment which could not be isolated from the initial construct was observed when pECPG3 was digested with .

Third construct

The 10 kb and 2.5 kb components of pNJR-12 were successfully ligated, in effect eliminating the 4.5 kb pBE-5 component. After ligation of these two components, an attempt was made to ligate the third component, pYHBA1, at the *Bam*H1 site. The

ligation mixture was electroporated into *E.coli* S17 and transformants were selected. However, miniprep procedures and gel analysis of 42 transformants failed to reveal a plasmid with the characteristic 5 kb band indicative of pYHBA1.

Matings

The pECPG3/*P. gingivalis* 33277 matings are summarized in Tables 1and 2. Matings with *P. gingivalis* using *E. coli* strains containing pECPG3 did not confer erythromycin resistance to *P. gingivalis*, as evidenced by the lack of growth on plates supplemented with erythromycin. Neither S17 nor XL-1 Blue/R751 *E. coli* cells were effective in transferring the plasmid. Although one colony was observed on erythromycin-supplemented media in mating #12, this was determined to be a contaminant. Growth was noted on aerobic plates supplemented with ampicillin (L-AMP, Appendix A) in matings #11 and 15, suggesting presence of the ampicillin resistance component of pECPG3 in *E. coli*. However, ampicillin resistant *E. coli* colony forming units (CFU) were much less than *E. coli* grown on non-selective media, suggesting a loss of the plasmid from *E. coli* during mating.

E. coli-E. coli matings using pECPG3 are summarized in Tables 3 and 4. Transfer of pECPG3 was observed with both S17 and XL1-Blue/R751 *E. coli* donor cells. Rate of transfer varied from 0 to 10^9 CFU, and results are similar for both donor types. Matings in which the donor : recipient ratio was increased to 1.5:1 show increased rates of transfer.

Matings utilizing existing vector systems are summarized in Table 5. The pNJR-12 vector was able to confer tetracycline resistance to *P. gingivalis* 33277, although at a low frequency. Increasing donor : recipient cell ratio to 2.5:1 in experiments 31 and 32

did not result in an increased rate of transfer. The number of *P. gingivalis* colonies on the selective media (LRBB-Tet, Appendix A) was approximately six orders of magnitude less than the non-selective media (LRBB, Appendix A). Transposons R751A and R751::Ω4 were also limited in their ability to transfer information to *P. gingivalis* 33277. Matings 6, 9, 10 , and 13 showed no transconjugants, while matings 14, 17-1 and 17-2 produced transconjugants on the order of 10^1-10^3 CFU.

Ampicillin resistance

P. gingivalis was successfully cultured in broths containing up to 0.5 μg/ml ampicillin. At concentrations higher than 0.5 μg/ml, however, *P. gingivalis* was either killed or competitively inhibited by contaminants. In addition, darkfield microscopy revealed morphologic changes in ampicillin resistant *P. gingivalis* cells. These mutants presented as long, almost filamentous rods significantly different from the short rods normally observed in the wild type.

Discussion

Goal

The intent of this study was to construct an *E. coli* - *P. gingivalis* conjugal shuttle vector. This proposed vector was to contain the following components:

1. pYHBA1, isolated from *P. asacchrolytica*,[36, 120] which is intended to allow stable replication in *P. gingivalis*.
2. An origin of replication from pBluescript (Stratagene, Inc.) that functions in *E. coli*.
3. An ampicillin resistance gene carried on pBluescript (Stratagene, Inc.) that will function in *E. coli* to allow selection of plasmid-carrying cells.
4. An erythromycin resistance gene, *erm*F, originally isolated from the *Bacteroides fragilis* plasmid pBF4.[104, 115] This will allow selection of transconjugants in *P. gingivalis*.
5. An origin of transfer, RP4 *ori*T, from plasmid RP4,[2] which will allow mobilization of the plasmid out of *E. coli*.

Because the proposed shuttle vector is transferred by conjugation as single-stranded DNA, it should be able to circumvent *P. gingivalis* endonucleases. These enzymes recognize the methylation pattern of DNA bases and cleave foreign DNA. Because the conjugal vector is transferred in a single-strand, the complementary strand is synthesized and methylated by *P. gingivalis*, and thus is not recognized as foreign. This offers a substantial advantage over vectors introduced by electroporation, which introduces double-stranded DNA into the target cells.[19] Yoshimoto and coworkers found that their vector system could be successfully electroporated into *P. gingivalis* only after the recipient cells had been chemically mutagenized, presumably to disable their endonucleases.[119] This compromises the results of any subsequent complementation

experiments, as it would be impossible to ascertain which additional genes had been inactivated in the mutagenized *P. gingivalis* strains.

An additional goal was to insert pYHBA1 into a previously described vector system, pNJR-12.[58] It was theorized that addition of the pYHBA1 fragment to pNJR-12 would enhance stability and replication in *P. gingivalis*.

First construct

After ligation of the pYHBA1, *ori*T, Amp^r, and *erm*F components, the first construct was electroporated into *E. coli* and plated on L-XICT media supplemented with ampicillin. This media allows antibiotic selection and color screening of transformants. In addition to transformants containing the desired construct, other transformant colonies may occur from cells which have undergone spontaneous mutation or "illegitimate" ligation (i.e. containing the ampicillin resistance gene but not the entire construct). In order to ascertain the presence of all plasmid components, plasmid DNA was extracted, restricted, and evaluated by agarose gel electrophoresis for the fragment sizes corresponding to the restriction map. Thirty-one transconjugants were isolated and evaluated by miniprep, restriction and agarose gel electrophoresis. However, agarose gel electrophoresis of digests with *Eco*RI did not demonstrate a 0.4 kb band consistent with the presence of *ori*T, which is required to transfer the plasmid out of *E. coli*. It was concluded that that this fragment was not part of the plasmid. This would produce a plasmid that could confer ampicillin resistance and color selection, but could not be mobilized out of *E. coli* due to the lack of *ori*T. Steric hinderance due to the proximity of the *Pst*I and *Eco*RI restriction sites on pDC1-12 may have inhibited the double digestion of this plasmid which is necessary for subsequent ligation with the other components of

the construct. As a result, the existing fragments most likely closed on themselves rather than joining with the smaller *ori*T fragment. The manufacturer (Stratagene, La Jolla CA) indicates that *Eco*RI displays decreased efficiency in double digests with *Pst*I when the recognition sites are in close proximity (in pDC1-12, they are adjacent). Inefficient restriction by *Pst*I may have resulted in a lack of viable sites on pDC1-12 for the *ori*T fragment to ligate to. This would result in a construct lacking the *ori*T fragment. Because the experimental design required inclusion of the *ori*T fragment, this construct was abandoned.

Second construct (pECPG3)

The components for the second construct were similar to the first, but with sufficient space between the restriction sites to allow proper digestion of all fragments needed for the ligation. After ligation and electroporation, eight transformants were selected on the basis of growth on selective media. Analysis by gel electrophoresis revealed that at least one transformant contained a restriction pattern predicted by the plasmid map. It was theorized that the *ori*T fragment would allow mobilization out of *E. coli*, while the origins of replication in the pBluescript and pYHBA1 would allow replication in both species. The erythromycin resistance gene would allow selection of *P. gingivalis* transconjugants in *E. coli* - *P. gingivalis* matings, while the ampicillin resistance gene would allow selection of transconjugants in *E. coli* -*E. coli* matings. *E. coli* cells containing pECPG3 were mated with both *P. gingivalis* and *E. coli* cells to determine whether the plasmid would function as designed.

The *E. coli* -*P. gingivalis* matings were unsuccessful in transferring pECPG3 from *E. coli* to *P. gingivalis* (Tables 1 and 2). To determine whether the plasmid or the

mating procedure was at fault, previously described vectors were used to test the mating protocol. Successful matings utilizing several *E. coli* vector systems and *P. gingivalis* 33277 (i.e. R751::Ω4, pNJR-12, Tables 5 and 6), verified that transfer to *P. gingivalis* was possible. Efficiency of these matings was similar to those previously reported for Tn4351 and pNJR-12.[35, 58, 89] Frequency of transfer for pNJR-12 to *P. gingivalis* was 10^{-6}-10^{-7}. Maley and coworkers reported a transfer frequency of 3.1 x 10^{-6} for pNJR-12 using a donor : recipient ratio of 1.25:1; this frequency could be increased by two orders of magnitude with a donor : recipient ratio of 262:1. The authors also reported a transfer frequency of 2.5 x 10^{-5} for pNJR-5 into *P. gingivalis* at 12:1 donor : recipient ratio, compared to 1.3 x 10^{-7} at a ratio of 1:3. As pNJR-12 is a conjugal shuttle vector, the successful transfer validates the mating protocol. Using the pE5-2 conjugal shuttle vector, Progulske-Fox and coworkers (1989) reported a transfer frequency of 1.4 - 2.0 x 10^{-7} into *P. gingivalis*.[79] Given the variation in frequency of transfer, it is possible that our system did not employ a sufficient donor : recipient ratio to allow transfer of pECPG3. Our initial matings were conducted with a 1:1 donor : recipient ratio, which was later increased to 1.5:1 and 2.5:1 (Appendix C). Previous studies have shown substantial increases in transfer frequency with increased donor cell concentration. In our case, it is possible that pECPG3 was less efficient than pNJR-12 or pE5-2, and a higher donor : recipient ratio was required for conjugal transfer and replication in transconjugants.

Transfer of R751::Ω4 to *P. gingivalis* was also successful. R751::Ω4 is a suicide vector that is able to transfer into *P. gingivalis*, but does not replicate within *P. gingivalis*. R751::Ω4 has been used to insert transposon Tn4351 into the *P. gingivalis* chromosome.

Transfer frequency into *P. gingivalis* was approximately 10^{-6}, which is consistent with previous studies of this vector system.[35, 89] The successful transfer associated with the R751::Ω4 system may be due in part to the fact that, unlike pNJR-12, the transposon (Tn4351) is maintained in the chromosome and does not have to replicate independently within *P. gingivalis*.

Another potential flaw in the transfer of pECPG3 is the inability of the plasmid to mobilize out of the donor cells. Failure of the donor strain to mobilize the plasmid would result in no transconjugants observed on selective media (i.e. they would lack the antibiotic resistance gene). The pECPG3 *E. coli* - *E. coli* matings (Tables 3 and 4) were conducted to evaluate mobilization out of the *E. coli* donor strains S17 and XL1-Blue/R751 and into *E. coli* strain RVN. Results of these matings show that the plasmid could be mobilized out of either S17 or XL1-Blue/R751 with varying degrees of success. Depending on the donor : recipient ratio, pECPG3 was transferred out of *E. coli* S17 at frequencies ranging from 10^{-3} to 10^{-10}. Using R751 as a donor strain resulted in transfer frequencies on the order of 10^{-5}. Ultimately, these results rule out a lack of mobilization of pECPG3 out of *E. coli* as a cause for failure.

In addition to the components of the mating system, the technique and protocol were also evaluated. Variations of the mating protocol are listed in Appendix C. Manipulation of donor : recipient cell ratio, duration of aerobic incubation, and centrifugation versus spot mating were all applied to enhance plasmid transfer. For example, experiment #13 (Table 5) utilizing R751::Ω4 and *P. gingivalis* 33277, utilized a 1.5:1 donor : recipient ratio and resulted in a higher frequency of transfer than previous matings using a 1 : 1 ratio (experiments #9, #13). However, experiment #12 (pECPG3

and *P. gingivalis* 33277) also used a1.5:1 donor : recipient ratio, but still resulted in no transconjugants.

Experiments 15, 16, and 17 varied the aerobic incubation period, with the matings designated "-1" incubated for 1.5 hours aerobic and those designated "-2" incubated for 18 hours (Appendix C). Although the results show that this adjustment improved transfer frequency of R751::Ω4 to *P. gingivalis*, it actually decreased transfer of pNJR-12 to *P. gingivalis*. Since pNJR-12 is a conjugal vector system, it was theorized that pECPG3 may behave in similar fashion. However, results showed no transconjugants with either incubation period (Table 1).

After validating the donor cells, recipient cells, cell ratio, and incubation times, it was hypothesized that pECPG3 might be unstable in the *E.coli* donor cells in the absence of selective pressure. Because the matings were carried out on LRBB without erythromycin (Appendix A), it is possible that the plasmid was transferred to *P. gingivalis* but not maintained in the absence of selective pressure during subsequent cell divisions. Additionally, the plasmid may not have been maintained in sufficient numbers in *E. coli* to allow conjugal transfer. In fact, the results of matings 11, 12, 15, 16, and 19-30 reveal marked reduction of ampicillin-resistant donor cell colonies compared to cells plated on non-selective media. These results imply that the plasmids were not being maintained in *E. coli* in the absence of selective pressure. A possible solution to this problem would be to carry out the matings in the presence of ampicillin. Since wild-type *P. gingivalis* 33277 is sensitive to ampicillin, development of an ampicillin-resistant strain of *P. gingivalis* would allow matings on ampicillin-supplemented media, thus providing selective pressure on *E. coli* to retain pECPG3. Although this strategy would

still involve using a mutant rather than a wild-type strain as a recipient, the level of mutation is not comparable to that introduced by chemical mutagens as in the Yoshimoto study.[119] Attempts to isolate an ampicillin-resistant *P. gingivalis* mutant were unsuccessful at ampicillin concentrations higher than 0.5 μg/ml. Higher concentrations resulted in death of the cells and/or contamination by other ampicillin-resistant species.

Third construct

The aim of the third construct was to enhance the stability of the pNJR-12 vector by inserting the pYHBA1 plasmid. Previous studies have used the pNJR-12 vector for complementation experiments, but were unable to sustain the vector in subcultures of *P. gingivalis* W83.[73] The component of pNJR-12 responsible for replication in *P. gingivalis* is a 4.1 kb *Bacteroides* plasmid fragment, pB8-51. It was hypothesized that removal of the pB8-51 fragment from pNJR-12 and addition of the 5 kb pYHBA1 plasmid would improve maintenance and replication within *P. gingivalis*, and consequently improve the efficiency of the vector system. However, we were unable to successfully ligate pYHBA1/pNJR-12. Although the fragments were isolated, ligated, and electroporated into *E. coli*, gel analysis of transformant DNA revealed a 12.5 kb plasmid with restriction fragments consistent with the 10 kb RSF1010 vector/kanamycin resistance fragment and 2.5 kb tetracycline resistance fragment . No evidence of a 5 kb fragment characteristic of pYHBA1 was seen after digestion with *Bam*H1. These results suggest false positives, i.e. the pNJR-12 fragment closed back on itself, reforming a 12.5 kb plasmid rather than integrating the 5 kb pYHBA1 fragment. The proposed pNJR-12/pYHBA1 construct is also a large plasmid (17.5 kb). This may have contributed to its failure to ligate, its inefficient electroporation, or its instability in *E. coli*.

Conclusions

1. A chimeric shuttle vector (pECPG3) was constructed. This vector contains all the elements theoretically necessary for conjugal transfer from *E. coli* to *P. gingivalis*, as well as elements for selection and maintenance in *P. gingivalis*.

2. Transfer to and/or maintenance of pECPG3 in *P. gingivalis* was unsuccessful, although pECPG3 could be mobilized from *E. coli* to *E. coli* by conjugal transfer.

3. Analysis of the mating protocol utilized in this study reveals that genetic material (pNJR-12 and R751::Ω4) could be transferred from *E. coli* to *P. gingivalis*. Therefore, pECPG3 is either not accepted by or not maintained in *P. gingivalis* at the concentrations of cells used. Further experiments may benefit by increasing of the donor : recipient cell ratio, which may permit a higher rate of transfer for pECPG3.

4. pECPG3 was unstable in *E. coli*. This may account for the lack of success in mobilizing pECPG3 from *E. coli* to *P. gingivalis*. Future mating attempts may benefit from the use of an ampicillin-resistant recipient strain. This strategy would allow matings to be carried out under selective conditions which would necessitate maintenance of the pECPG3 plasmid in *E. coli* donor cells.

5. The pYHBA1 plasmid was not successfully ligated to the RSF1010 vector and Tet^r components of the pNJR-12 vector.

Table 1: pECPG3-*P. gingivalis* matings
Donor: *E. coli* S17 **Recipient: *P. gingivalis* 33277**

Experiment	Colony forming units	Colony type
1	1.2×10^9	*P. gingivalis* recipient cells
	0	Transconjugants
2	1.0×10^9	*P. gingivalis* recipient cells
	0	Transconjugants
3	3.7×10^9	*P. gingivalis* recipient cells
	0	Transconjugants
4	4.4×10^9	*P. gingivalis* recipient cells
	0	Transconjugants
5	2.1×10^8	*P. gingivalis* recipient cells
	0	Transconjugants
11	3.9×10^9	*E. coli* donor cells
	1.0×10^4	Amp^r *E. coli* donor cells
	1.7×10^{10}	*P. gingivalis* recipient cells
	0	Transconjugants
12	7.0×10^9	*E. coli* donor cells
	0	Amp^r *E. coli* donor cells
	1.7×10^8	*P. gingivalis* recipient cells
	0	Transconjugants
	10	Contaminants
15-1	3.9×10^6	*E. coli* donor cells
	2.0×10^5	Amp^r *E. coli* donor cells
	1.2×10^8	*P. gingivalis* recipient cells
	0	Transconjugants
15-2	7.7×10^8	*E. coli* donor cells
	1.0×10^5	Amp^r *E. coli* donor cells
	1.2×10^8	*P. gingivalis* recipient cells
	0	Transconjugants

Table 2: pECPG3-*P. gingivalis* matings
Donor: *E. coli* XL-1 Blue with plasmid R751A **Recipient: *P. gingivalis* 33277**

Experiment	Colony forming units	Colony type
16-1	1.3×10^9	*E. coli* donor cells
	0	Amp^r *E. coli* donor cells
	3.8×10^9	*P. gingivalis* recipient cells
	0	Transconjugants
16-2	3.6×10^9	*E. coli* donor cells
	0	Amp^r *E. coli* donor cells
	$3.0\ 10^7$	*P. gingivalis* recipient cells
	0	Transconjugants

Table 3: ECPG3-*E. coli* matings
Donor: *E. coli* S17 **Recipient: *E. coli* RVN**

Experiment	Colony forming units	Colony type
20A	3.2×10^9	*E. coli* cells
	8.2×10^6	Amp^r *E. coli* cells
	6.3×10^5	Amp^r, Nalidixic acid^r transconjugants
20B	1.5×10^{11}	*E. coli* cells
	0	Amp^r *E. coli* cells
	16	Amp^r, Nalidixic acid^r transconjugants
21	2.8×10^9	*E. coli* cells
	4.0×10^5	Amp^r *E. coli* cells
	1.0×10^4	Amp^r, Nalidixic acid^r transconjugants
23	2.6×10^9	*E. coli* cells
	9.0×10^5	Amp^r *E. coli* cells
	0	Amp^r, Nalidixic acid^r transconjugants
25	3.0×10^9	*E. coli* cells
	8.0×10^5	Amp^r *E. coli* cells
	9.0×10^5	Amp^r, Nalidixic acid^r transconjugants
27	5.2×10^9	*E. coli* cells
	4.7×10^5	Amp^r *E. coli* cells
	2.8×10^4	Amp^r, Nalidixic acid^r transconjugants
29	8.1×10^8	*E. coli* cells
	3.8×10^7	Amp^r *E. coli* cells
	2.4×10^7	Amp^r, Nalidixic acid^r transconjugants

Table 4: pECPG3-*P. gingivalis* matings
Donor: *E. coli* XL-1 Blue with plasmid R751A **Recipient: *E. coli* RVN**

Experiment	Colony forming units	Colony type
19A	2.3×10^9	*E. coli* cells
	9.0×10^6	Amp^r *E. coli* cells
	3.2×10^6	Amp^r, Nalidixic $acid^r$ transconjugants
19B	4.2×10^9	*E. coli* cells
	0	Amp^r *E. coli* cells
	5.0×10^4	Amp^r, Nalidixic $acid^r$ transconjugants
22	2.9×10^9	*E. coli* cells
	0	Amp^r *E. coli* cells
	0	Amp^r, Nalidixic $acid^r$ transconjugants
24	3.5×10^9	*E. coli* cells
	0	Amp^r *E. coli* cells
	1.4×10^2	Amp^r, Nalidixic $acid^r$ transconjugants
26	1.4×10^{10}	*E. coli* cells
	0	Amp^r *E. coli* cells
	0	Amp^r, Nalidixic $acid^r$ transconjugants
28	2.0×10^9	*E. coli* cells
	$> 3 \times 10^5$	Amp^r *E. coli* cells
	$> 3 \times 10^5$	Amp^r, Nalidixic $acid^r$ transconjugants
30	4.8×10^9	*E. coli* cells
	7.2×10^5	Amp^r *E. coli* cells
	1.4×10^5	Amp^r, Nalidixic $acid^r$ transconjugants

Table 5: Matings utilizing previously described vectors and *P. gingivalis* 33277

Experiment	Donor	Colony forming units	Colony type
10	R751::Ω4	1.1×10^7 9.0×10^9 1.0×10^8 0 10	Amp^r *E. coli* donor cells Tet^r *E. coli* donor cells *P. gingivalis* recipient cells Transconjugants Contaminants
13	R751A	3.1×10^8 5.0×10^7 0	Trimethoprim^r *E. coli* donor cells *P. gingivalis* recipient cells Transconjugants
14	R751A	1.1×10^9 1.5×10^9 6×10^3	Tet^r *E. coli* donor cells *P. gingivalis* recipient cells Transconjugants
17-1	R751::Ω4	1.7×10^9 1.4×10^9 50	Tet^r *E. coli* donor cells *P. gingivalis* recipient cells Transconjugants
17-2	R751::Ω4	1.2×10^7 9.9×10^8 18	Tet^r *E. coli* donor cells *P. gingivalis* recipient cells Transconjugants
18-1	pNJR-12	3.0×10^7 9.0×10^9 3.9×10^3	Kan^r *E. coli* donor cells *P. gingivalis* recipient cells Transconjugants
18-2	pNJR-12	3.3×10^8 3.0×10^9 0	Kan^r *E. coli* donor cells *P. gingivalis* recipient cells Transconjugants
31	pNJR-12	8.5×10^6 5.0×10^7 30	Kan^r *E. coli* donor cells *P. gingivalis* recipient cells Transconjugants
32	pNJR-12	1.2×10^8 3.0×10^7 20	Kan^r *E. coli* donor cells *P. gingivalis* recipient cells Transconjugants

Figure 1: Schematic representation of complementation

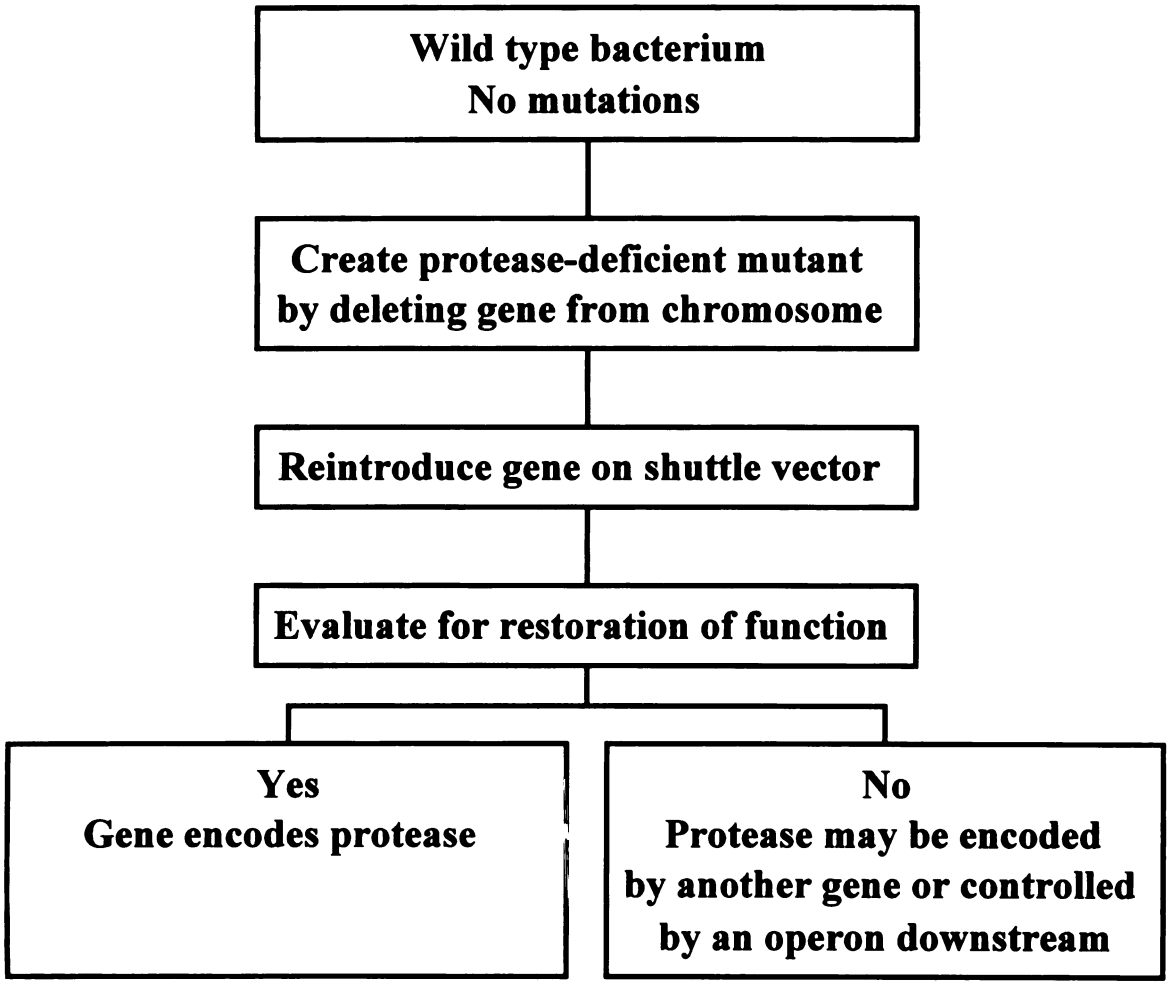


Figure 2: pYHBA1

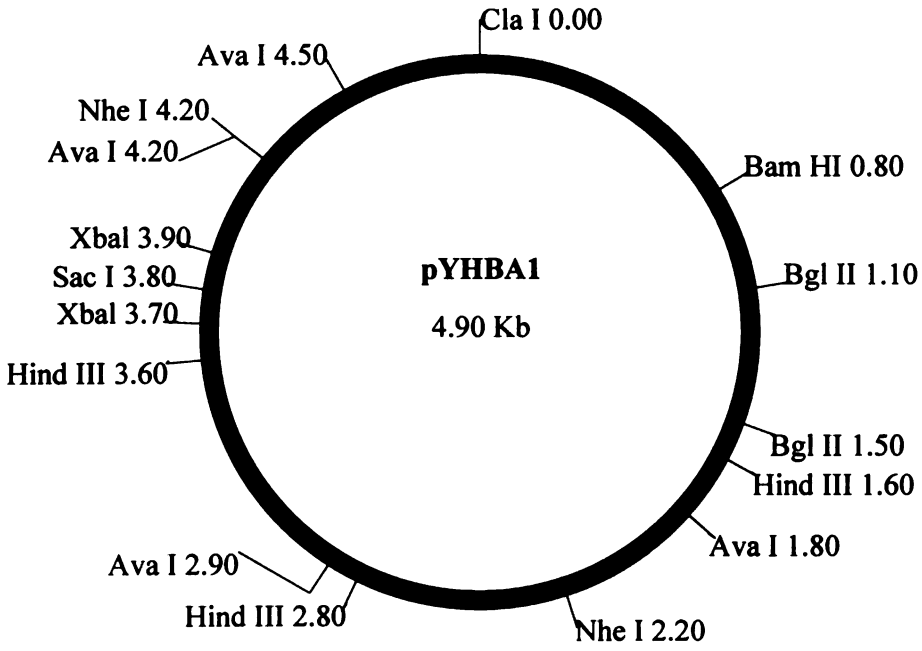


Figure 3: pDC1-12

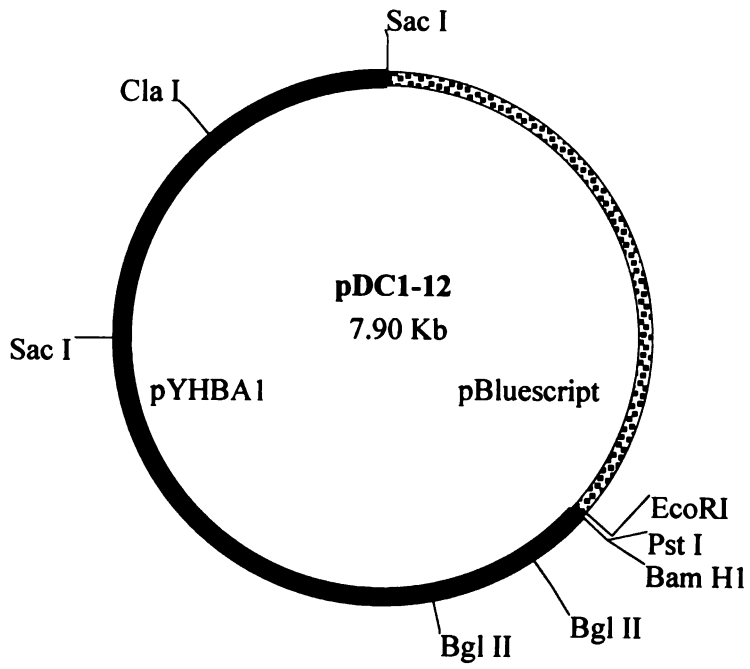


Figure 4: Initial construct

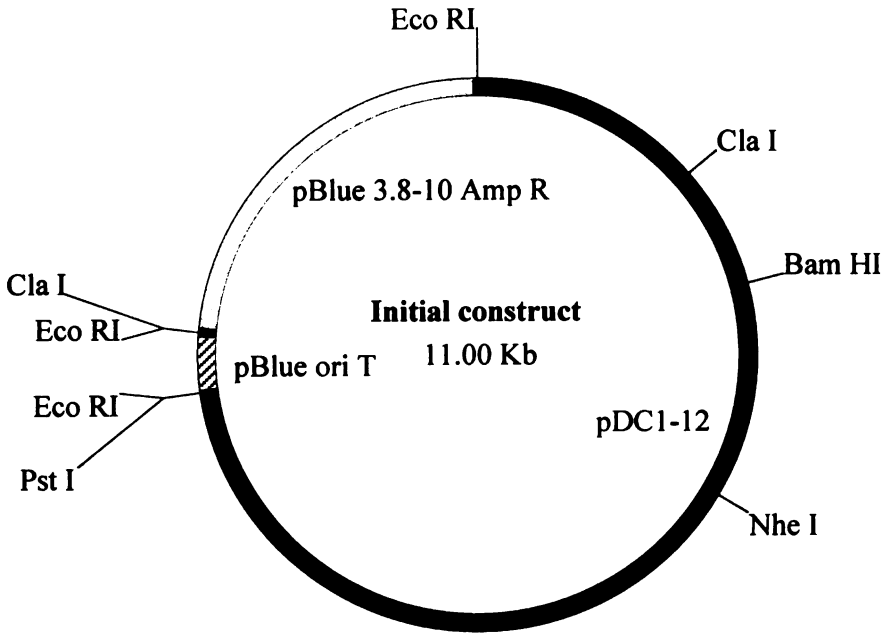


Figure 5: pECPG3

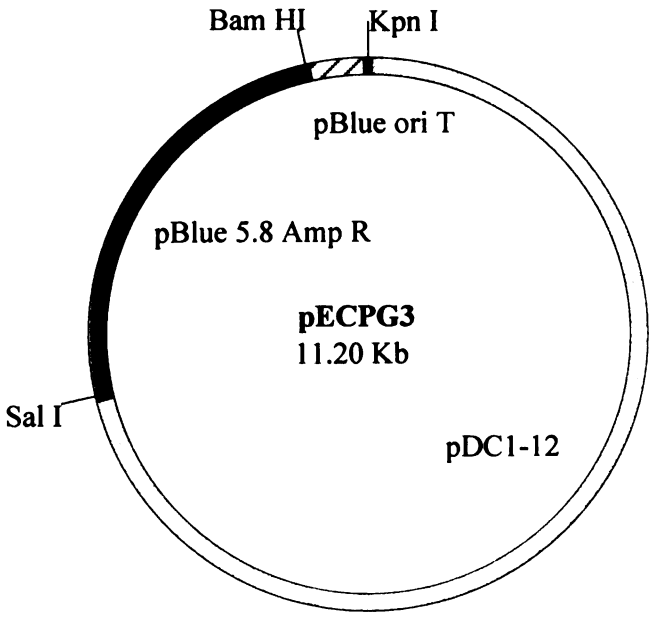


Figure 6: pNJR-12

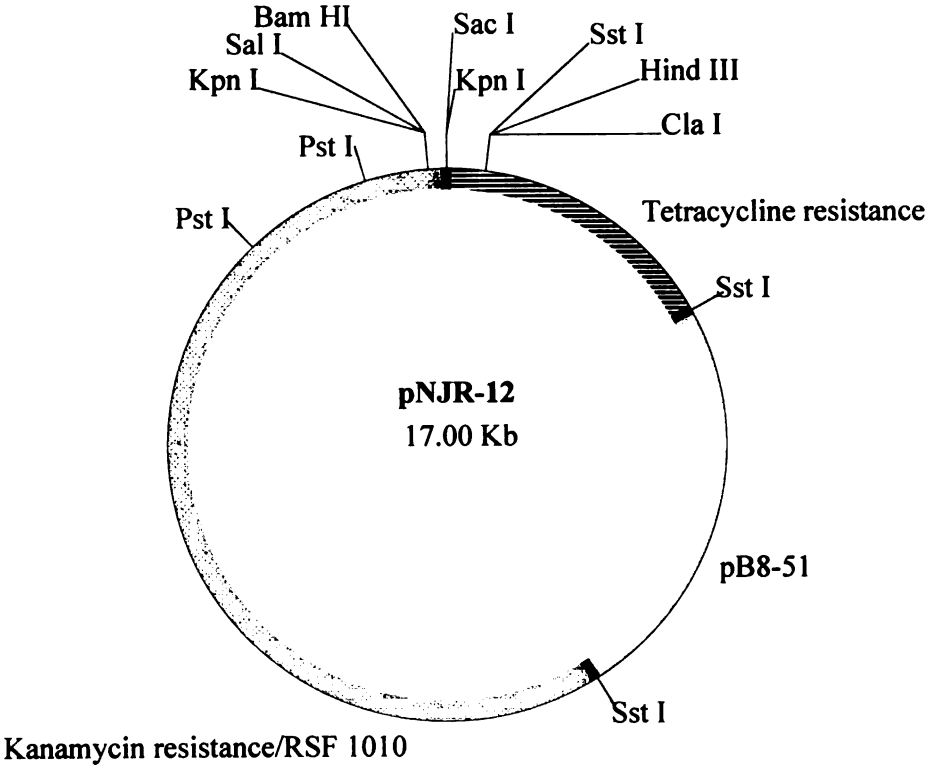


Figure 7: pNJR-12/pYHBA1 construct

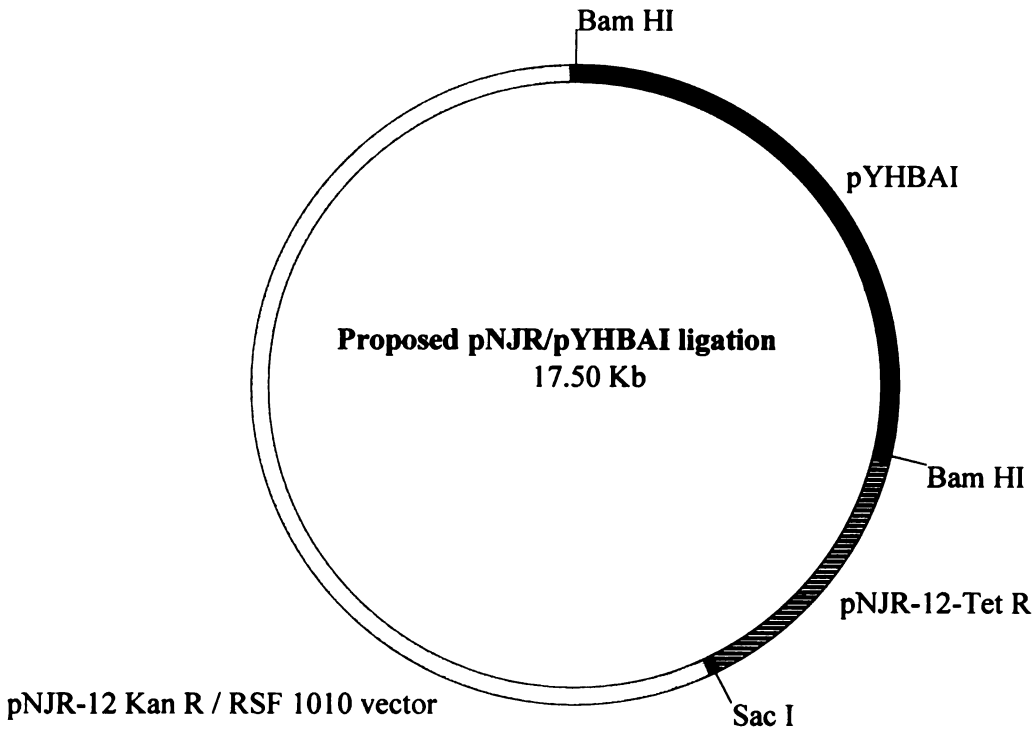
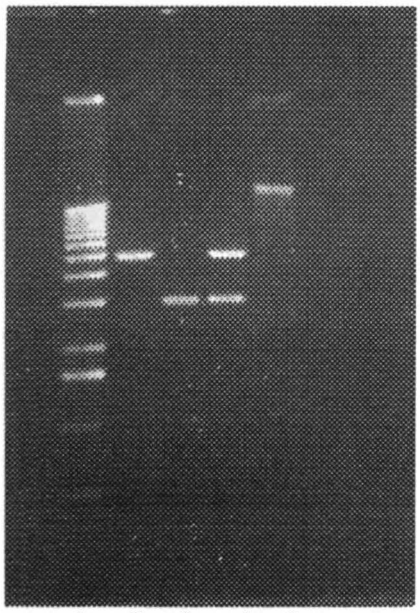


Figure 8
Agarose gel electrophoresis, pDC1-12

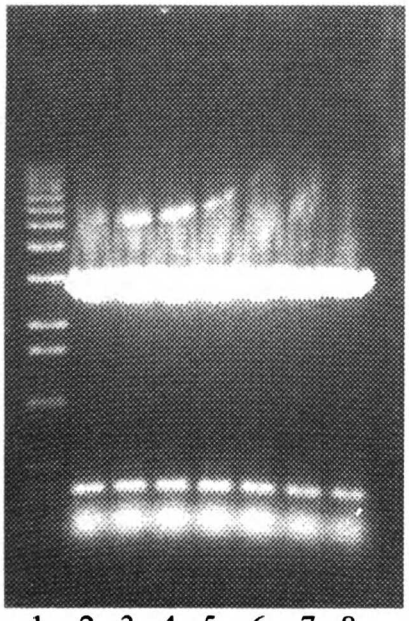


Lane	Contents
1	1 kb DNA standard*
2	pYHBA1 (*Bam*HI cut)
3	pBluescript (*Bam*HI cut)
4	pDC1-12 (*Bam*HI cut)
5	pDC1-12 (uncut)

Lanes 2 and 3 exhibit bands at 5 and 3 kb, respectively, consistent with the fragments used in construction of the chimeric plasmid. Digestion of the chimeric plasmid with *Bam*HI yields the two component fragments.

*Refer to Appendix B

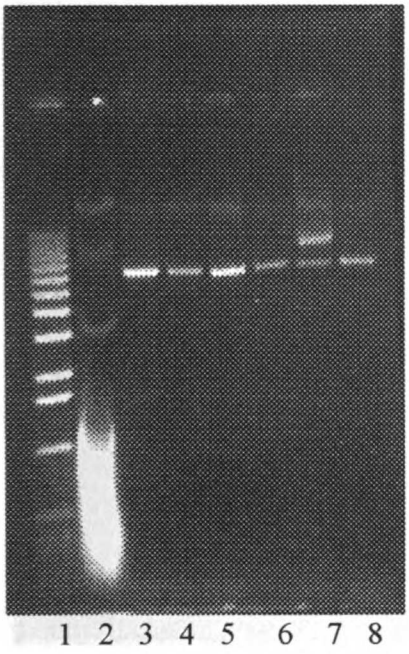
Figure 9
Isolation of pBlue *ori*T



Lane	Contents
1	1 kb DNA standard
2-8	pBlue *ori*T (*Cla*I/*Pst*I cut)

The 0.4 kb band containing the *ori*T gene is evident (second line from the bottom of gel). This band was excised for DNA extraction by QIAEX. The remaining bands were discarded.

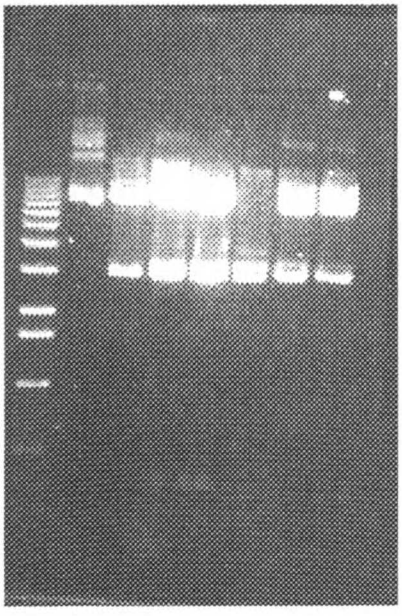
Figure 10
Agarose gel electrophoresis, first construct



Lane	Contents
1	1 kb DNA standard
2	Plasmid DNA from white transformant colony (uncut)
3-8	Plasmid DNA from 6 white transformant colonies digested with *Eco*RI

Digestion with *Eco*RI should yield bands at 2.8, 8, and 0.4 kb per the construct map. This gel shows bands at approximately 8 kb, suggesting false positive results.

Figure 11
Gel electrophoresis identifcation of transformants containing pECPG3

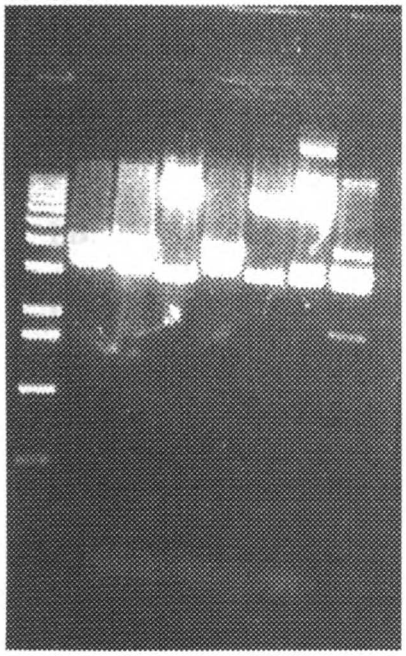


Lane	Contents
1	1 kb DNA standard
2	Plasmid DNA from white trasformant colony (uncut)
3-8	Plasmid DNA from 6 white transformant colonies (*Eco*RI cut)

Lanes 3 and 4 exhibit bands at 0.4, 2.8, and 8 kb, suggesting the presence of all components involved in the ligation.

Figure 12

Gel analysis of transformant DNA constructs



1 2 3 4 5 6 7 8

Lane	Contents
1	1 kb DNA standard
2	Transformant 1 (*Cla*I cut)
3	Transformant 2 (*Cla*I cut)
4	Transformant 2 (*Pst*I cut)
5	Transformant 5 (*Cla*I cut)
6	Transformant 5 (*Pst*I cut)
7	Transformant 7 (*Eco*RI cut)
8	Transformant 8 (*Eco*RI cut)

Consistent with the plasmid map of pECPG3, digestion with *Pst*I yields bands at 2.8 and 8.4 kb and *Cla*I yields bands at 3.2, 3.8, and 4.2 kb. *Eco*RI should yield bands at 0.4, 2.8, and 8 kb. Constructs 7 and 8 do not exhibit this pattern, suggesting "illegitimate" combination of fragments during ligation.

Appendix A: Media

Aerobic media:

SOB broth: 2% tryptone, 0.5% yeast extract, 0.0005 % NaCl

SOC broth: SOB broth supplemented with 1 ml 2 M glucose and 1 ml Mg salt solution per 100 ml

LB: Luria-Bertani agar

L-XICT: Luria-Bertani agar supplemented with 80 μg/ml X-Gal, 5 mM IPTG, 100 μg/ml carbenicillin, and 10 μg/ml tetracycline.

L-KAN: Luria-Bertani agar supplemented with 50 μg/ml kanamycin

L-AMP: Luria-Bertani agar supplemented with 100 μg/ml ampicillin

Tri-Thy: Luria-Bertani agar supplemented with 50 μg/ml thymine and 200 μg/ml trimethoprim

NANA: Luria-Bertani agar supplemented with 100 μg/ml ampicillin and 30 μg/ml nalidixic acid

Anaerobic media:

sTSB broth: 3% trypticase soy broth and 0.25% yeast extract supplemented with 0.5 ml 50 mg % hemin per 100 ml, 0.1 ml 500 mg % menadione per 100 ml, and 1 ml 1% dithiothreitol per 100 ml.

LRBB: Laked rabbit blood Brucella agar

LRBB-GENT: Laked rabbit blood Brucella agar supplemented with 50 μg/ml gentamycin

LRBB ERY-GENT: Laked rabbit blood Brucella agar supplemented with 10 μg/ml erythromycin and 50 μg/ml gentamycin

LRBB-AMP: Laked rabbit blood Brucella agar supplemented with 100 μg/ml ampicillin

Appendix B: Molecular techniques

Miniprep and maxiprep procedures:

These procedures are a modification of the technique described by Godson and Vapnek (Godson and Vapnek 1972).

Miniprep: Cells were grown overnight in 3-4 ml broth and harvested by centrifugation at 14,000 rpm for 2 minutes. The cells were then washed with 1 ml 0.25 M Tris and resuspended in 50 mM Tris/10% sucrose. Lysozyme in 0.25 M Tris was then added, followed by 0.25 M EDTA and 20% sodium dodecyl sulfate. The lysates were incubated on ice for 15 minutes. After this incubation, 5 M NaCl was added, and the cell debris was separated by centrifugation at 14,000 rpm for one hour. The supernatant containing the plasmid DNA was extracted twice with a 25:24:1 misture of phenol:chloroform/isoamyl alcohol, then once with 24:1 choroform:isoamyl alcohol. The DNA was precipitated with one-fourth volume of 10 M ammonium acetate, then an equal volume of isopropanol. The extracted DNA was then incubated overnight at 4° C. After incubation, the DNA was separated by centrifugation at 14,000 rpm for 15 minutes, washed with 70% ethanol, and resuspended in 1X Tris-EDTA buffer.

Maxiprep: The reagents and protocol for this extraction are similar to the miniprep procedure. 500 ml of cell culture is harvested in the initial step. The volumes of the reagents are increased accordingly. The separation of DNA and cell debris after lysis is accomplished by ultracentrifugation at 25,000 rpm at 0°C instead of 14,000 rpm. After incubation, the plasmid DNA is purified by cesium chloride density gradient ultracentrifugation.

DNA Extraction: DNA was extracted from agarose gels using the QIAEX system (Qiagen, Valencia, CA) per manufacturer's protocol.

Ligation: Ligations were carried out using T4 DNA ligase (BRL, Berkeley, CA). 20 µl of each plasmid were combined with 12 µl 5X ligation buffer, 3 µl T4 DNA ligase, and 5 µl dH_20. The mixture was incubated overnight at 16°C. The ligation mixture was then precipitated with 15 µl ammonium acetate and 75 µl isopropanol and incubated overnight. After incubation, the ligation product was washed twice with 70% ethanol and resuspended in dH_20.

Electroporation: 40 µl of electrocompetent XL1-Blue *E. coli* cells were added to 10 µl of plasmid suspension and electroporated at 100 ohms, 25 µ*f*, 10 kv using a BioRad Gene Pulser (BioRad, Hercules CA). A rate constant was recorded, and the electroporation mixture was incubated for one hour at 37°C. After incubation, the electroporation mixture was plated on selective media.

1 kb DNA standard: This standard displays bands of the following sizes in ascending order: 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0.

Appendix C: Mating protocols

Experiments 1-5: 2 ml of donor and recipient cells were harvested by centrifugation at 14,000 rpm for 2 minutes. The pellet was resuspended in 200 ml of SOB broth and spotted onto nutrient agar in 100 µl increments. The spots were incubated for 2 hours in an aerobic environment, then overnight in an anaerobic environment.

Experiments 15-18: Harvesting was similar to experiments 1-5. However, experiments designated with -1 were incubated for 2 hours aerobic before anaerobic incubation, and those designated -2 were incubated 18 hours aerobic before anaerobic incubation.

Experiments 19 and 20: Experiments designated A used cells harvested by centrifugation as described above. Those designated B used 50 µl of broth cultures of each cell type spotted together on a nutrient agar plate. The spots were incubated for 2 hours in an aerobic environment, then overnight in an anaerobic environment.

Experiments 21-26: 50 µl of broth cultures of each cell type were spotted together on a nutrient agar plate, similar to experiments 19B and 20B. The spots were incubated for 2 hours in an aerobic environment, then overnight in an anaerobic environment.

Experiments 27-30: 3 ml of donor cell suspensions and 2 ml of recipient cell cultures were harvested by centrifugation. The pellet was resuspended in 200 ml SOB broth and spotted onto nutrient agar in 100 µl increments. The spots were incubated for 2 hours in an aerobic environment, then overnight in an anaerobic environment.

Experiments 31: 5 ml of donor cell suspensions and 2 ml of recipient cell cultures were harvested by centrifugation. The pellet was resuspended in 200 ml SOB broth and spotted onto nutrient agar in 100 µl increments. The spots were incubated for 1 hours in an aerobic environment, then 36 hours in an anaerobic environment.

Experiment 32: 5 ml of donor cell suspensions and 2 ml of recipient cell cultures were harvested by centrifugation. The pellet was resuspended in 200 ml SOB broth and spotted onto nutrient agar in 100 µl increments. The spots were incubated for 40 hours in an aerobic environment, then 36 hours in an anaerobic environment.

After the initial anaerobic incubation of the mating spots, the spots were resuspended in 1 ml of sTSB, ten-fold serially diluted in sTSB, and 0.1 ml portions of undiluted and diluted suspensions were plated on appropriate selective and non-selective media.

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