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[54] **METHOD AND MATERIALS FOR INTRODUCING DNA INTO *PREVOTELLA RUMINICOLA***

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[73] Assignees: **The Board of Trustees of the University of Illinois, Urbana-Champaign, Illinois**, Urbana; **Biotechnology Research and Development Corporation**, Peoria, both of Ill.

[21] Appl. No.: **718,535**

[22] Filed: **Jun. 5, 1991**

[51] **Int. Cl.**[5] **C12N 1/21**; C12N 15/63; C12N 15/74

[52] **U.S. Cl.** **435/172.3**; 435/252.3; 435/320.1

[58] **Field of Search** 435/172.3, 252.3, 320.1

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Shoemaker, et al., *J. Bacteriol.*, 172:1694–1702 (1990).
Shoemaker, et al., *J. Bacteriol.*, 166:959–965 (1986).
Shoemaker, et al., *J. Bacteriol.*, 170:1651–1657 (1988).
Shoemaker, et al., *J. Bacteriol.*, 162:626–632 (1985).
Shoemaker, et al., *J. Bacteriol.*, 171:1294–1302 (1989).
Simon, et al., *Bio/Technology*, 1:784–791 (1983).
Smith, *J. Bacteriol.*, 169:4589–4596 (1987).
Smith, *J. Bacteriol.*, 164:294–301 (1985).
Sougakoff, et al., *FEMS Microbiol. Lett.*, 44:153–159 (1987).
Speer, et al., *J. Bacteriol.*, 170:1423–1429 (1988).
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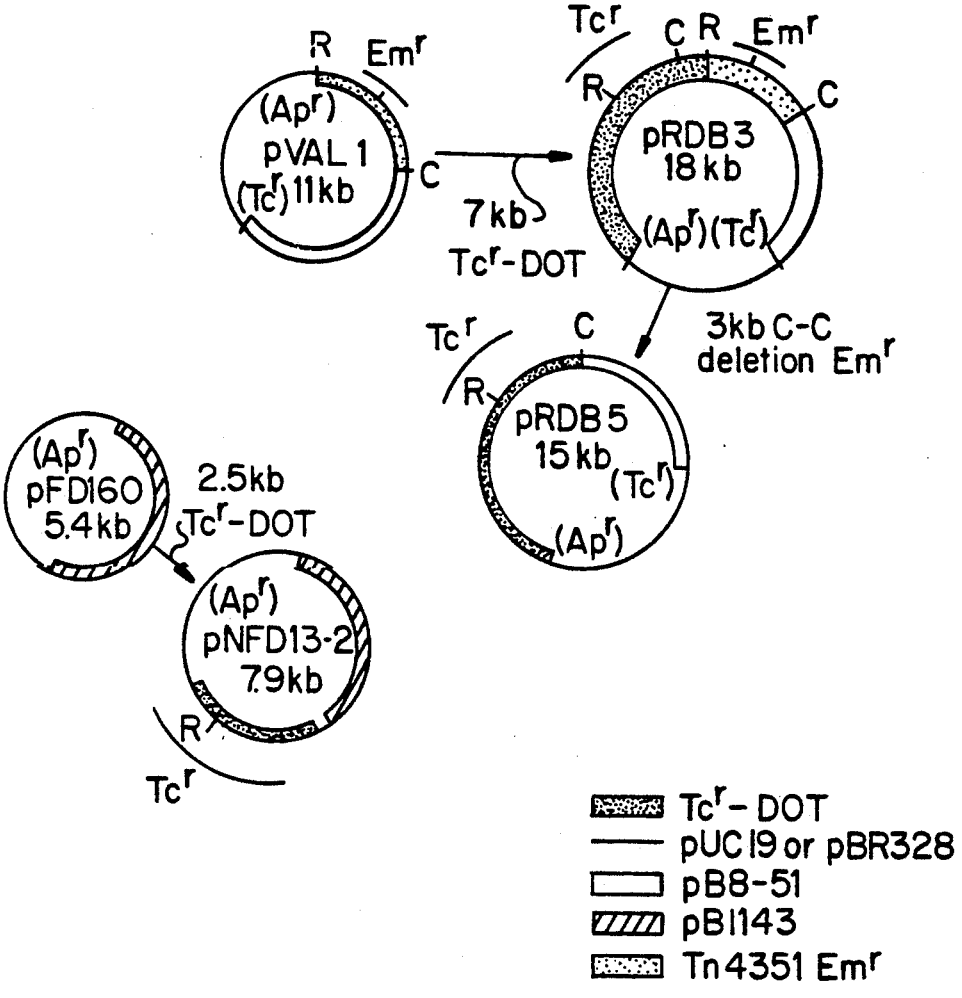
Attorney, Agent, or Firm—Willian Brinks Olds Hofer Gilson & Lione

[57] **ABSTRACT**

A method of introducing expressible heterologous DNA into *Prevotella ruminicola* is provided. The method involves conjugal transfer of a shuttle vector comprising the heterologous DNA operatively linked to a promoter functional in *P. ruminicola*. The invention also provides shuttle vectors for use in the method and *P. ruminicola* produced by the method. The invention further provides a tetracycline resistance gene of the TetQ class, or fragments thereof that confer tetracycline resistance, and a protein of the TetQ class that provides resistance to tetracycline by protecting ribosomes from tetracycline, or active fragments thereof. Finally, the invention provides a promoter functional in *P. ruminicola* and an engineered *P. ruminicola* comprising expressible foreign DNA.

12 Claims, 10 Drawing Sheets

FIG.1


R
Em^r
(Ap^r)
pVAL 1
(Tc^r) 11kb
C
7kb
Tc^r-DOT
Tc^r
C R
Em^r
R
C
pRDB3
18 kb
(Ap^r)(Tc^r)
3kb C-C
deletion Em^r
Tc^r
C
R
pRDB5
15 kb
(Tc^r)
(Ap^r)
(Ap^r)
pFD160
5.4kb
2.5kb
Tc^r-DOT
(Ap^r)
pNFD13-2
7.9kb
R
Tc^r
Tc^r-DOT
pUC19 or pBR328
pB8-51
pB1143
Tn4351 Em^r

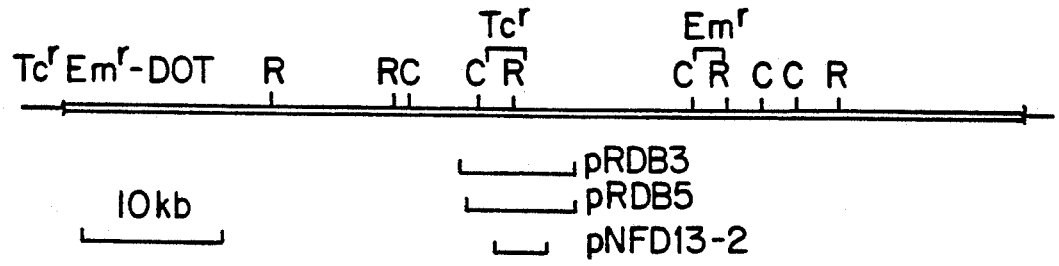

Tc^r Em^r-DOT
R
RC
Tc^r
C R
Em^r
C R C C R
10kb
pRDB3
pRDB5
pNFD13-2

FIG. 2A

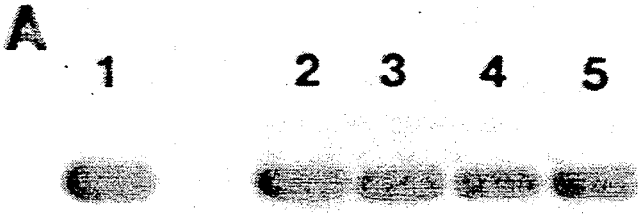


FIG. 2B

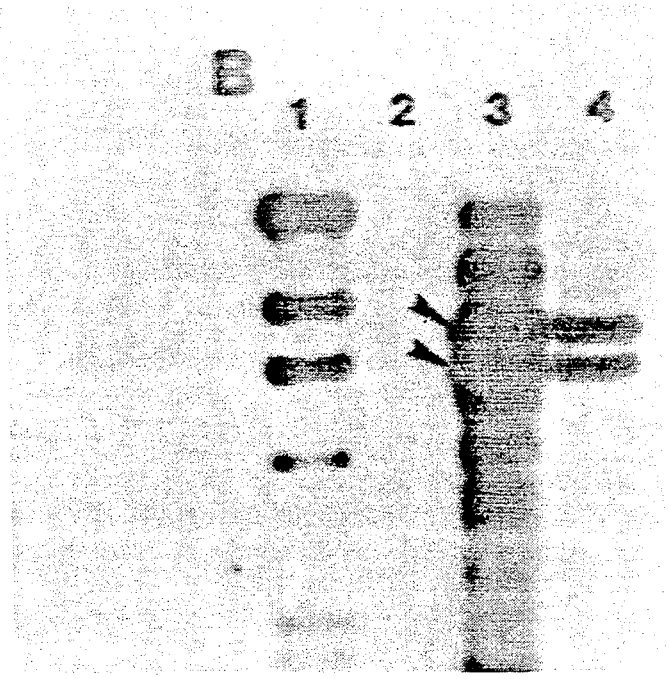
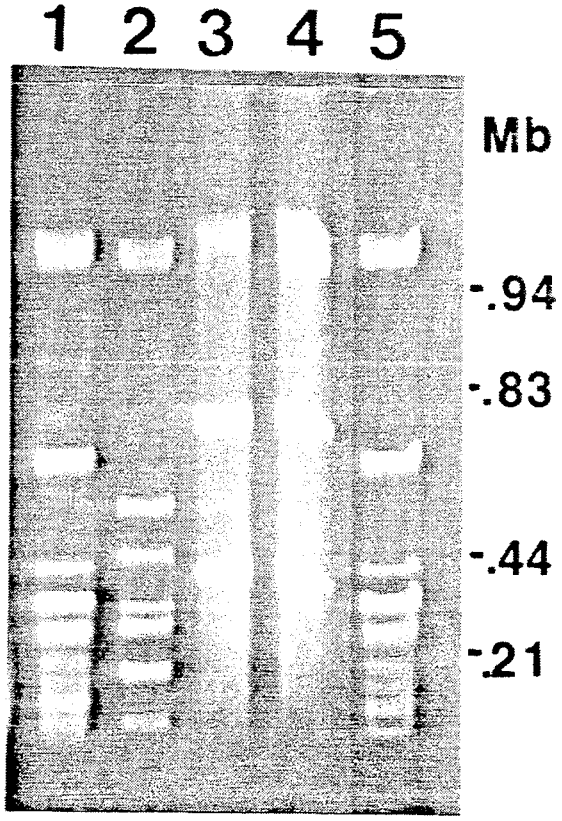


FIG. 3



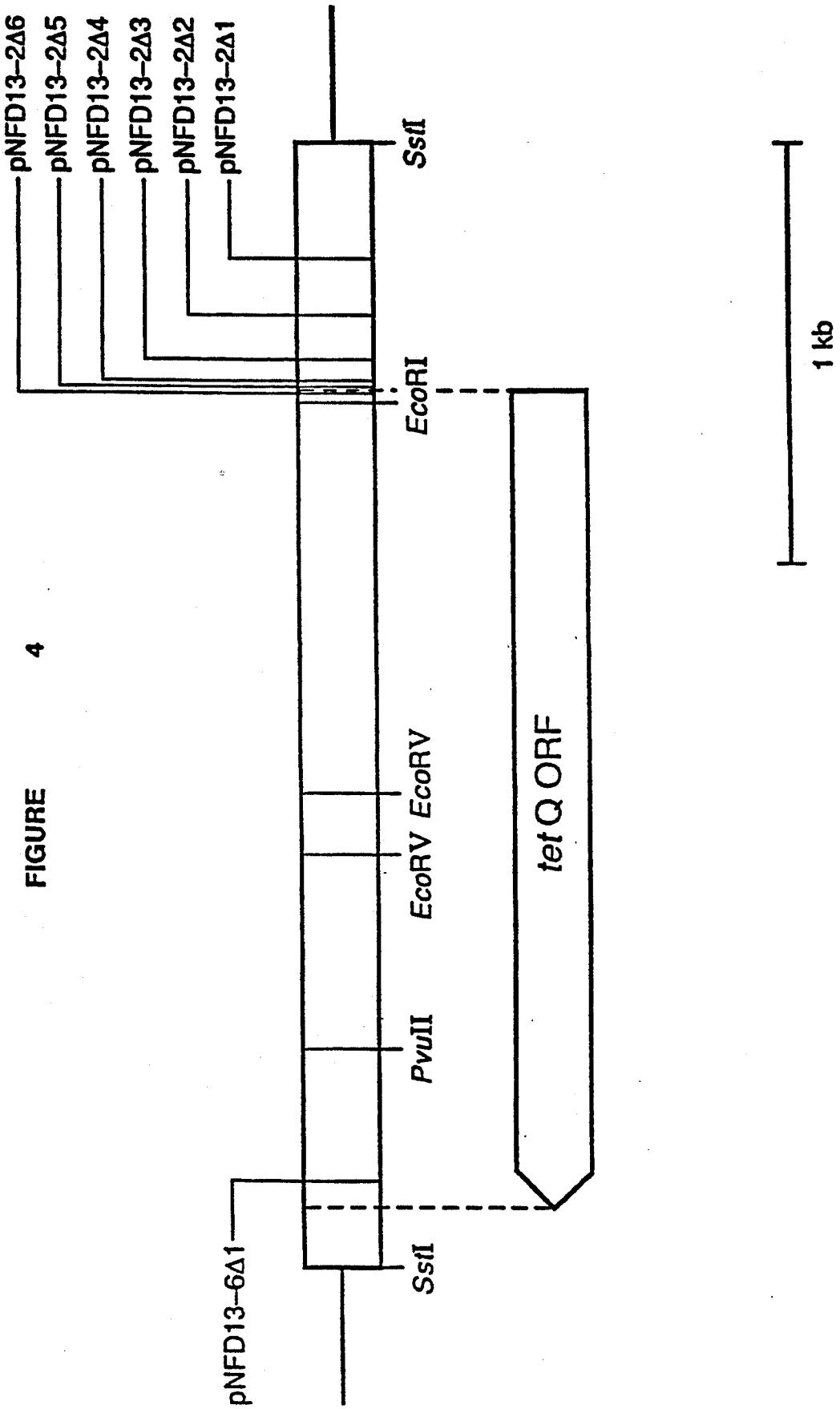

pNFD13-2Δ6
pNFD13-2Δ5
pNFD13-2Δ4
pNFD13-2Δ3
pNFD13-2Δ2
pNFD13-2Δ1
SstI
EcoRI
EcoRV
EcoRV
PvuII
SstI
pNFD13-6Δ1
tet Q ORF
1 kb

FIGURE 4

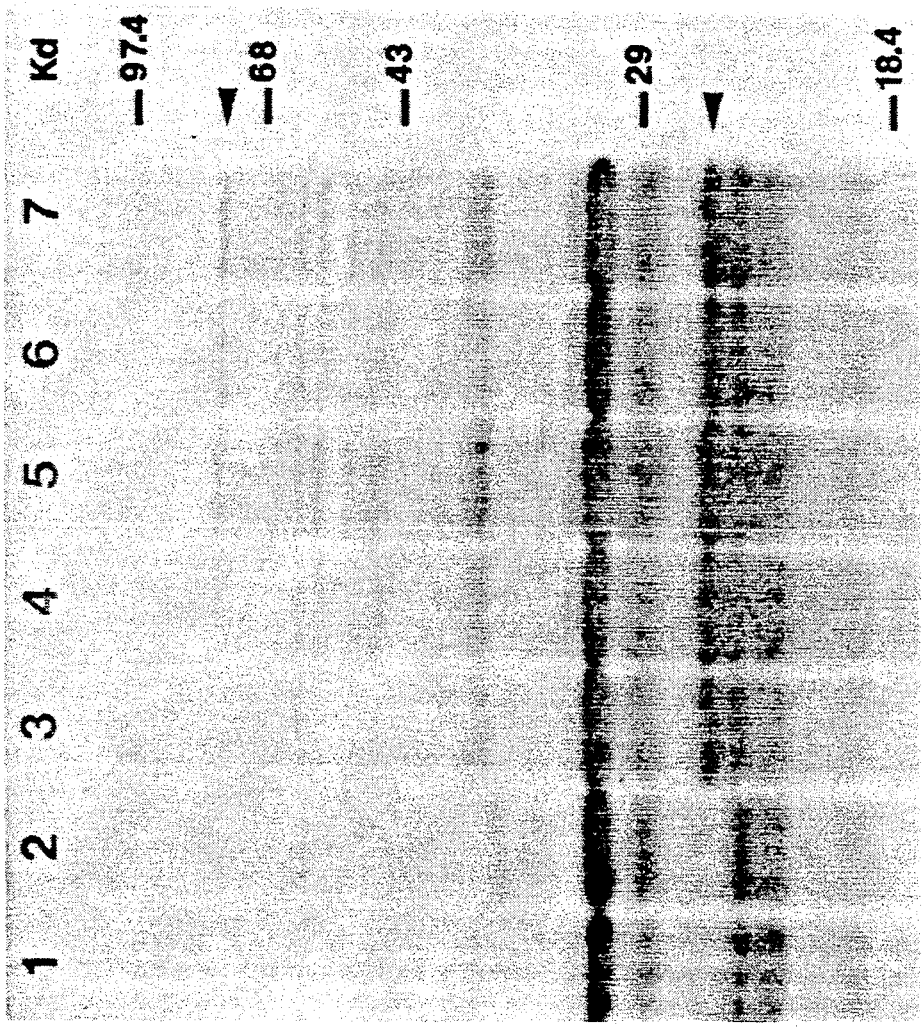
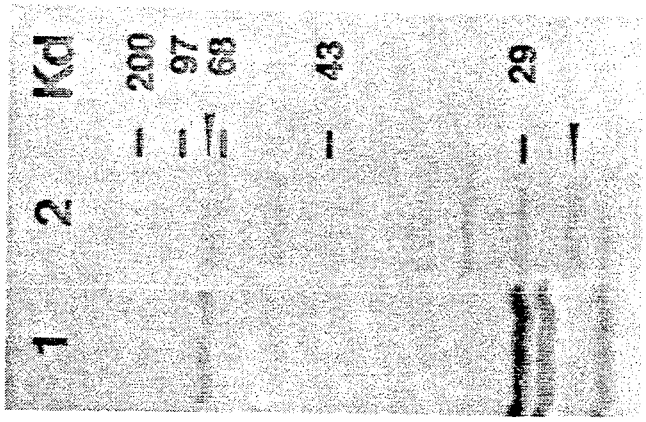


FIG. 5A

FIG. 5B



```
                0                                                      49
Bat-TetQ        MNIINLGILA  HIDAGKTSVT  ENLLFASGAT  EKCGCVDNGD  TITDSMDIEK
Caj-TetO        MKIINLGILA  HVDAGKTTLT  ESLLYTSGAI  AELGSVDEGT  TRTDTMNLER
Stp-TetM        MKIINIGVLA  HVDAGKTTLT  ESLLYNSGAI  TELGSVDRGT  TKTDNTLLER
Consensus       MkIINlGiLA  HvDAGKTtlT  EsLLy.SGAi  .elGsVD.Gt  T.TD.m.lEr
                   $ +  +   $ $+$$++ +  + +  +     + +  +     +    +

                50                                                     99
Bat-TetQ        RRGITVRAST  TSIIWNGVKC  NIIDTPGHMD  FIAEVERTFK  MLDGAVLILS
Caj-TetO        QRGITIQTAV  TSFQWEDVKV  NIIDTPGHMD  FLAEVYRSLS  VLDGAVLLVS
Stp-TetM        QRGITIQTAI  TSFQWKNTKV  NIIDTPGHMD  FLAEVYRSLS  VLDGAILLIS
Consensus       qRGITiqta.  TSfqW..vKv  NIIDTPGHMD  FlAEVyRsls  vLDGAVLl.S
                 $+$$$      +           +++$+$$$ $  +  ++ ++     ++$+$

                100                                                    149
Bat-TetQ        AKEGIQAQTK  LLFNTLQKLQ  IPTIIFINKI  DRAGVNLERL  YLDIKANLSQ
Caj-TetO        AKDGIQAQTR  ILFHALQIMK  IPTIFFINKI  DQEGIDLPMV  YREMKAKLSS
Stp-TetM        AKDGVQAQTR  ILFHALRKIG  IPTIFFINKI  DQNGIDLSTV  YQDIKEKLSA
Consensus       AKdGiQAQTr  iLFhaLqk..  IPTIfFINKI  Dq.GidL..v  Y.diKakLS.
                   $  $                 + + + $$    $  +                +

                150                                                    199
Bat-TetQ        DVLFMQNVVD  GSVYPVCSQT  YIKEEYKEFV  CNHDDNILER  YLADSEISPA
Caj-TetO        EIIVKQKVGQ  HPHINVTDND  DMEQ..WDAV  IMGNDELLEK  YMSGKPFKMS
Stp-TetM        EIVIKQKVEL  HPNMRVMNFT  ESEQ..WDMV  IEGNDYLLEK  YTSGKLLEAL
Consensus       ei..kQkV..  hp...V...t  ..eq..wd.V  i.gnD.lLEk  Y.sgk.....
                   +                                      ++     + +

                200                                                    249
Bat-TetQ        DYWNTIIALV  AKAKVYPVLH  GSAMFNIGIN  ELLDAITS.F  ILPPASVSNR
Caj-TetO        ELEQEENRRF  QNGTLFPVYH  GSAKNNLGTR  QLIEVIASKF  YSSTPEGQSE
Stp-TetM        ELEQEESIRF  HNCSLFPVYH  GSAKNNIGID  NLIEVITNKF  YSSTHRGQSE
Consensus       eleqee..rf  .n..lfPVyH  GSAknNiGi.  .LievItskF  ysst..gqse
                +                  ++  +++  + +

                250                                                    299
Bat-TetQ        LSSYLYKIEH  DPKGHKRSFL  KIIDGSLRLR  DVVRINDSEK  FIKIKNLKTI
Caj-TetO        LCGQVFKIEY  SEKRRRFVYV  RIYSGTLHLR  DVIRISEKEK  .IKITEMYVP
Stp-TetM        LCGKVFKIEY  SEKRQRLAYI  RLYSGVLHLR  DPVRISEKEK  .IKITEMYTS
Consensus       Lcg.vfKIEy  seKr.r..y.  riysG.LhLR  DvvRIsekEK  .IKItemyt.
                   +++                 $ ++$ +           +

                300                                                    349
Bat-TetQ        NQGREINVDE  VGANDIAIVE  DMDDFRIGNY  LGAEPCLIQG  ..LSHQHPAL
Caj-TetO        TNGELYSSDT  ACSGDIVILP  N.DVLQLNSI  LGNEILLPQR  KFIENPLPMI
Stp-TetM        INGELCKIDK  AYSGEIVILQ  N.EFLKLNSV  LGDTKLLPQR  ERIENPLPLL
Consensus       .nGel...D.  a.sgdIvIl.  n.d.l.lns.  LG.e.lLpQr  ..ienplP.l
                            +                      +            + +
```

FIGURE 6A

```
                350                                                      399
Bat-TetQ        KSSVRPDRPE  ERSKVISALN  TLWIEDPSLS  FSINSYSDEL  EISLYGLTQK
Caj-TetO        QTTIAVKKSE  QREILLGALT  EISDCDPLLK  YYVDTTTHEI  ILSFLGNVQM
Stp-TetM        QTTVEPSKPQ  QREMLLDALL  EISDSDPLLR  YYVDSATHEI  ILSFLGKVQM
Consensus       qttv.p.kpe  qRe.ll.AL.  eisd.DPlL.  yyvds.thEi  ilsflG.vQm
                            +    ++        ++                      +

                400                                                      449
Bat-TetQ        EIIQTLLEER  FSVKVHFDEI  KTIYKERPVK  KVNKIIQIEV  PPNPYWATIG
Caj-TetO        EVICAILEEK  YHVEAEIKEP  TVIYMERPLR  KAEYTIHIEV  PPNPFWASVG
Stp-TetM        EVTCALLQEK  YHVEIEIKEP  TVIYMERPLK  KAEYTIHIEV  PPNPFWASIG
Consensus       EvicalLeEk  yhVe.eikEp  tvIYmERPlk  KaeytIhIEV  PPNPfWAsiG
                            +      +    + + +

                450                                                      499
Bat-TetQ        LTLEPLPLGT  GLQIESDISY  GYLNHSFQNA  VFEGIRMSCQ  SGLHGWEVTD
Caj-TetO        LSIEPLPIGS  GVQYESRVSL  GYLNQSFQNA  VMEGVLYGCE  QGLYGWKVTD
Stp-TetM        LSVAPLPLGS  GVQYESSVSL  GYLNQSFQNA  VMEGIRYGCE  QGLYGWNVTD
Consensus       Ls.ePLPlGs  GvQyES.vSl  GYLNqSFQNA  VmEGirygCe  qGLyGW.VTD
                   +   +    +           +           +   +        ++ +

                500                                                      549
Bat-TetQ        LKVTFTQAEY  YSPVSTPADF  RQLTPYVFRL  ALQQSGVDIL  EPMLYFELQI
Caj-TetO        CKICFEYGLY  YSPVSTPADF  RLLSPIVLEQ  ALKKAGTELL  EPYLHFEIYA
Stp-TetM        CKICFKYGLY  YSPVSTPADF  RMLAPIVLEQ  VLKKAGTELL  EPYLSFKIYA
Consensus       cKicF.yglY  YSPVSTPADF  R.L.PiVleq  aLkkaGtelL  EPyL.Feiya
                 +     + +      +    +                +     +  ++

                550                                                      599
Bat-TetQ        PQAASSKAIT  DLQKMMSEIE  DISCNNEWCH  IKGKVPLNTS  KDYASEVSSY
Caj-TetO        PQEYLSRAYH  DAPRYCADIV  STQIKNDEVI  LKGEIPARCI  QEYRTDLTYF
Stp-TetM        PQEYLSRAYN  DAPKYCANIV  DTQLKNNEVI  LSGEIPARCI  QEYRSDLTFF
Consensus       PQeylSrAy.  Dapkyca.Iv  dtq.kN.evi  lkGeiParci  qeYrsdlt.f
                +           +      +                   +         +

                600                                                  645
Bat-TetQ        TKGLGIFMVK  PCGYQITKGG  YSDNIRMNEK  ..DKLLFMFQ  KSMSSK
Caj-TetO        TNGQGVCLTE  LKGYQPAIGK  FICQPRRPNS  RIDKVRHMFT  S
Stp-TetM        TNGRSVCLTE  LKGYHVTTGE  PVCQPRRPNS  RIDKVRYMFN  KIT
Consensus       TnG.gvclte  lkGYq.t.G.  ..cqpRrpns  riDKvr.MF.  k
                + +            +
```

FIGURE 6B

FIGURE 7A

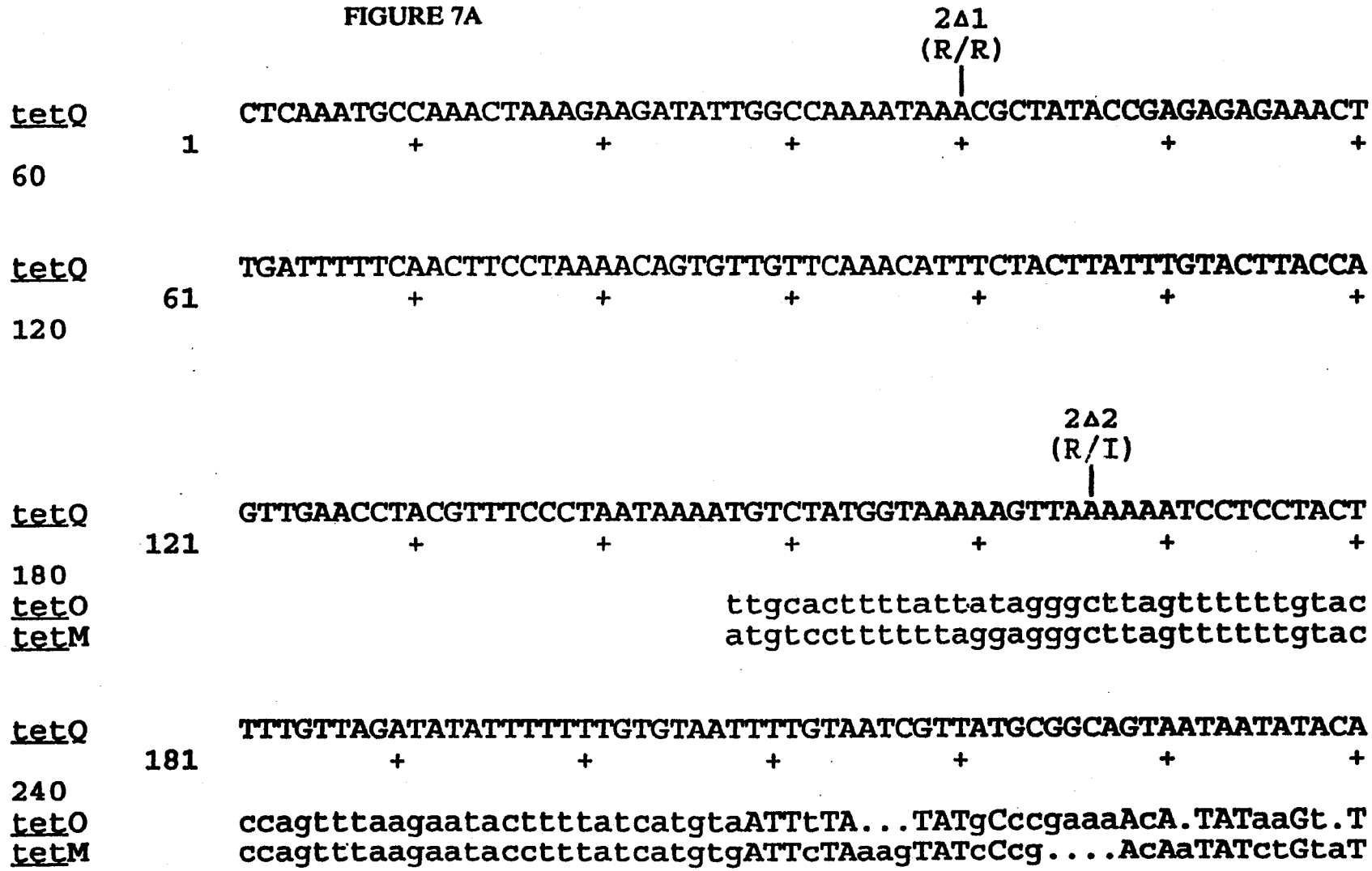
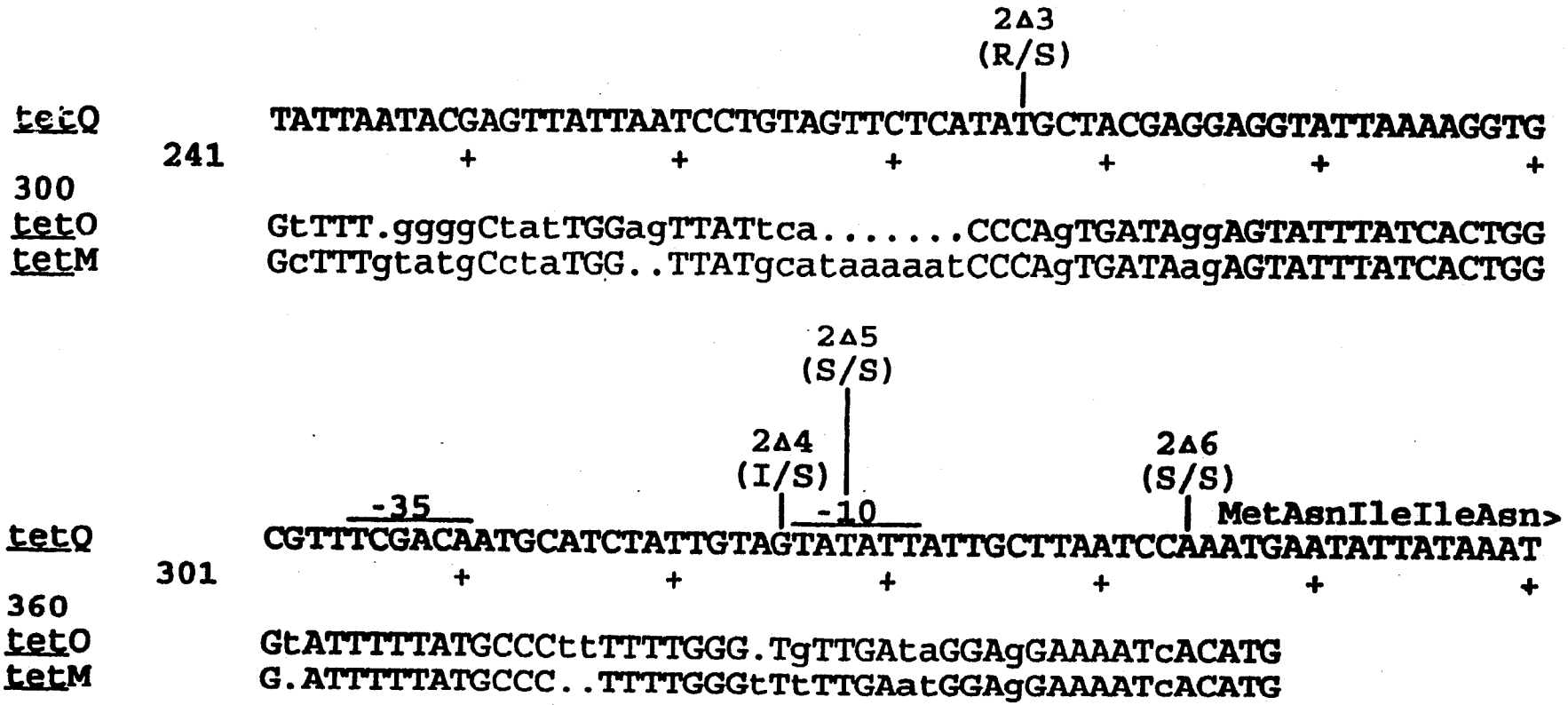

2Δ1
(R/R)
tetO CTCAAATGCCAAACTAAAGAAGATATTGGCCAAAATAAACGCTATACCGAGAGAGAAACT
1 + + + + + +
60
tetO TGATTTTTCAACTTCCTAAAACAGTGTTGTTCAAACATTTCTACTTATTTGTACTTACCA
61 + + + + + +
120
2Δ2
(R/I)
tetO GTTGAACCTACGTTTCCCTAATAAAATGTCTATGGTAAAAAGTTAAAAAATCCTCCTACT
121 + + + + + +
180
tetO ttgcacttttattatagggcttagtttttgtac
tetM atgtccttttttaggagggcttagtttttgtac
tetO TTTGTTAGATATATTTTTTTGTGTAATTTTGTAATCGTTATGCGGCAGTAATAATATACA
181 + + + + + +
240
tetO ccagtttaagaatactttatcatgtaATTtTA...TATgCccgaaaAcA.TATaaGt.T
tetM ccagtttaagaatacctttatcatgtgATTcTAaagTATcCcg....AcAaTATctGtaT

FIGURE 7B


2Δ3
(R/S)
tetQ
TATTAATACGAGTTATTAATCCTGTAGTTCTCATATGCTACGAGGAGGTATTAAAAGGTG
241
300
tetO
GtTTT.ggggCtatTGGagTTATtca.....CCCAgTGATAggAGTATTTATCACTGG
tetM
GcTTTgtatgCctaTGG..TTATgcataaaaatCCCAgTGATAagAGTATTTATCACTGG
2Δ5
(S/S)
2Δ4
(I/S)
2Δ6
(S/S)
-35
-10
MetAsnIleIleAsn>
tetQ
CGTTTCGACAATGCATCTATTGTAGTATATTATTGCTTAATCCAAATGAATATTATAAAT
301
360
tetO
GtATTTTTATGCCCttTTTTGGG.TgTTGAtaGGAgGAAAATcACATG
tetM
G.ATTTTTATGCCC..TTTTGGGtTtTTGAatGGAgGAAAATcACATG

METHOD AND MATERIALS FOR INTRODUCING DNA INTO *PREVOTELLA RUMINICOLA*

This invention was made with government support provided by Grant No. 59 32U4-7-119 awarded by the United States Department of Agriculture. The government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates generally to methods and materials for the genetic manipulation of *Prevotella ruminicola.* This invention also relates to a novel class of tetracycline resistance genes.

BACKGROUND OF THE INVENTION

A. Bacteroides and Prevotella

Bacteroides is a genus of Gram negative, obligately anaerobic bacteria found in the gastrointestinal tracts of humans and animals. These bacteria function in metabolizing a wide range of carbohydrates. In humans, Bacteroides account for approximately 25% of the bacteria in the colon.

Prevotella ruminicola is a species of Gram negative, obligately anaerobic bacteria found in the rumen of cattle. *P. ruminicola* ferment carbohydrates such as hemicellulose, cellobiose, and starch and aid digestion and degradation of polysaccharides. *P. ruminicola* was previously classified as a member of the genus Bacteroides (*Bacteroides ruminicola*) because it has some characteristics associated with human colonic Bacteroides However, recent investigations showed that *P. ruminicola* shared less than 5% DNA-DNA homology with the colonic Bacteroides species. More detailed biochemical analyses also suggested that it belonged in a separate genus, Prevotella [See Shah, et al., *Intl. J. Syst. Bacteriol.,* 40:205–208 (1990)].

Some progress has been made in connection with genetic manipulation of obligately anaerobic Bacteroides from the human colon. For example, shuttle vectors have been developed for use with some colonic Bacteroides which contain DNA from cryptic Bacteroides plasmids which are able to replicate in a number of different Bacteroides species [See Odelson, et al., *Plasmid,* 17:87–109 (1987); Salyers, et al., *Crit. Rev. Microbiol.,* 14:49–71 (1987); Valentine, et al., *J. Bacteriol..* 170:1319–1324 (1988)]. These vectors also contain sequences which allow them to replicate in *E. coli* and be mobilized out of *E. coli* by IncP plasmids. The IncP plasmids R751 and RP4 have been shown to mobilize DNA from *E. coli* to a variety of other species, including colonic Bacteroides species [See Salyers, et al., *Crit. Rev. Microbiol.,* 14:49–71 (1987); Shoemaker, et al., *J. Bacteriol.,* 166:959–965 (1986)]. One such *E. coli*-Bacteroides shuttle vector is pVAL-1 which contains cryptic Bacteroides plasmid pB8-51 [Valentine, et al., *J. Bacteriol.,* 170:1319–1324 (1988)].

Certain colonic Bacteroides strains have been found to harbor large self-transmissible elements carrying a tetracycline resistance ("Tc^{4}") gene which are referred to as "conjugal elements" or "Tc^{r} elements." Some of these Tc^{r} elements also carry a clindamycin-erythromycin resistance ("Em^{r}") gene and are referred to as "Tc^{r}Em^{r} elements." These elements are not plasmids, but are integrated into the host chromosome.

The Tc^{r} and Em^{r} genes from a conjugal Tc^{r}Em^{r} strain of Bacteroides, *Bacteroides thetaiotaomicron* DOT, have been cloned, along with regions of the element that include transfer genes [Shoemaker, et al., *J. Bacteriol.,* 171:1294–1302 (1989)]. The Tc^{r}Em^{r} element from *B. thetaiotaomicron* DOT has been designated "Tc^{r}Em^{r}-DOT."

These conjugal elements are able to transfer themselves from one colonic Bacteroides strain to another and to mobilize co-resident plasmids, not only from Bacteroides to Bacteroides, but also from Bacteroides to *E. coli* [See Odelson, et al., *Plasmid,* 17:87–109 (1987); Salyers, et al., *Crit. Rev. Microbiol.,* 14:49–71 (1987); Thomson, et al., *FEMS Microbiol. Letters,* 61:101–104 (1989); Stevens, et al., J. Bacteriol. 172:4271–4279 (1990)]. Thus, the Tc^{r} and Tc^{r}Em^{r} conjugal elements found in the colonic Bacteroides strains appear to be able to mediate mating pair formation between diverse genera of bacteria.

The conjugal element, Tc^{r}Em^{r} 12256, has been found to mobilize co-resident plasmids at high frequencies [See Valentine, et al., *J. Bacteriol.,* 170:1319–1324 (1988)]. Furthermore, the Tc^{r}Em^{r} 12256 element appears to exhibit constitutive transfer, as opposed to other Tc^{r} and Tc^{r}Em^{r} elements which require pre exposure to tetracycline to obtain maximum transfer frequencies.

Plasmid DNA has been introduced into some colonic Bacteroides using transformation techniques [See Salyers, et al., *CRC Clinical Reviews in Microbiology,* 14:49–71 (1987); Odelson, et al., *Plasmid* 17:87–109 (1987); Smith, *J. Bacteriol.,* 164:294–301 (1985)]. For instance, one colonic Bacteroides species has been transformed by electroporation [Thomson, et al., *FEMS Microbiol. Letters.* 61:101–104 (1989)]. An *E coli*-colonic Bacteroides shuttle vector, pDP1, was isolated from *Bacteroides uniformis* and electroporated into *B. uniformis* at a frequency of 10^{6} transporants per microgram of DNA. However, the same plasmid, when isolated from *E. coli* EM24, gave only 10^{3} transporants per microgram of DNA.

Standard methods, however, appear to be inadequate in several respects for the transformation of the colonic Bacteroides. For example, large plasmids are difficult to introduce into these species by transformation techniques. Best results are obtained when the plasmid DNA is less that 5 kbp in size. Also, to obtain good rates of transformation, the donor plasmid must be isolated from the same strain used as the recipient. The difficulties encountered in crossing species lines are believed to be due to the presence of restriction barriers. Also, successful transformation of many species of colonic Bacteroides has been sporadic [See Odelson, et al., *Plasmid,* 17:102 (1987)]. Clearly, much improvement is needed in transformation methods for colonic Bacteroides.

Despite progress in understanding the genetics of colonic Bacteroides, *P. ruminicola* is not well understood genetically. There have been some biochemical studies of polysaccharide utilization by *P. ruminicola,* and a xylanase gene from *P. ruminicola* has been cloned and expressed in *E. coli* [See Whitehead, et al., *Appl. Eviron. Microbiol.,* 55:893–896 (1989)].

Recently, a naturally-occurring plasmid carrying a gene coding for tetracycline resistance has been identified ("pRRI4") in *P. ruminicola* 223/M2/7. The pRRI4 plasmid was shown to transfer from *P. ruminicola* 223/M2/7 into *P. ruminicola* F101, but not into *P. ruminicola* 23, by conjugation [Flint, et al., *Appl. Environ. Microbiol.,* 54:855–860 (1988)].

It has also been reported that the pRRI4 plasmid can be introduced into *P. ruminicola* F101 by electroporation, but not into *P. ruminicola* 118B, M384, GA33 by this method [Thomson and Flint, *FEMS Microbiol. Letters,* 61:101–104 (1989)]. This article also reports that pRRI4 isolated from *P. ruminicola* could not be introduced into *B. uniformis,* a colonic Bacteroides, by electroporation. Thomson and Flint also discloses that the *E. coli*-colonic Bacteroides shuttle vector pDPI could not be introduced into *P. ruminicola* by electroporation. This was true whether pDPI was extracted from *B. uniformis* or *E. coli.*

From the above discussion, it is clear that, prior to the present invention, the genetic manipulation of *P. ruminicola* was not possible. Little was known about the genetics of *P. ruminicola,* making the use of vectors that could be manipulated and amplified in a known host, such as *E. coli,* highly desirable However, no shuttle vectors were known that could be used in *P. ruminicola.* Transformation and conjugal transfer of pRRI4 was possible, but pRRI4 cannot be used as a shuttle vector due to its relatively large size (19.5 kbp) and its inability to replicate in *E. coli.*

B. Tetracycline Resistance

Many bacteria, including strains of Bacteroides and Prevotella, possess tetracycline resistance genes. Three types of tetracycline resistance have been described and subdivided into classes defined by DNA-DNA hybridization.

The first type, tetracycline efflux, is mediated by a 40–50 kDa membrane protein which transports tetracycline out of the cell. Examples of this mode of resistance have been found in Gram-negative enterics [classes TetA–G; Aoki, *Micro. Sci.,* 5:219–223 (1988); Levy, *ASM News,* 54:418–421 (1988)] and some Gram-positive bacteria [classes TetK and TetL; Lacks, et al., *J. Mol. Biol.,* 192:753–765 (1986); McMurry, et al., *Antimicrob. Agents Chemother.,* 32:1646–1650 (1987)].

The second type of tetracycline resistance, ribosome protection, is mediated by a 72–75 kDa cytoplasmic protein which interacts with ribosomes and prevents inhibitory binding of tetracycline. Examples of this mode of resistance have been found in many Gram-positive and some Gram-negative bacteria [classes TetM and TetO; Burdett, *J. Bacteriol.,* 165:564–569 (1986); Manavathu, et al., *Gene,* 62:17–26 (1988); Sougakoff, et al., *FEMS Microbiol. Lett.,* 44:153–159 (1987)].

The third type of resistance, tetracycline modification, is mediated by a 44 kDa cytoplasmic protein which chemically inactivates tetracycline. The only known representative of this mode of resistance, class TetX, was orginally found in *B. fraqilis* [Speer and Salyers, *J. Bacteriol.,* 170:1423–1429 (1988)].

Two other Tc^r genes are known. TetN is an unsequenced streptococcal Tc^r which is reported to confer ribosome protection type resistance [Burdett, *J. Bacteriol,* 165:564–569 (1986)]. TetP is an uncharacterized Tc^r determinant from *Clostridium prefringens* [Abraham, et al., *Plasmid,* 19:113–120 (1988)].

SUMMARY OF THE INVENTION

The invention provides for the first time a method for the genetic manipulation of *Prevotella ruminicola.* In particular, the present invention provides a method for introducing heterologous DNA into *P. ruminicola.* The method comprises transforming *E. coli* with a shuttle vector comprising: a mobilization region which permits transfer of the shuttle vector from *E. coli* to a colonic Bacteroides species; a mobilization region which permits transfer of the shuttle vector from the colonic Bacteroides species to a *P. ruminicola:* and heterologous DNA operatively linked to a promoter functional in *P. ruminicola.* After transformation of the *E. coli* with the shuttle vector, the *E. coli* is contacted with the colonic Bacteroides species under conditions sufficient so that the shuttle vector is transferred from the *E. coli* to the colonic Bacteroides species. Finally, the colonic Bacteroides species containing the shuttle vector is contacted with the *P. ruminicola* under conditions sufficient so that the shuttle vector is transferred from the colonic Bacteroides species to the *P. ruminicola.*

The invention also comprises *P. ruminicola* produced by this method and a shuttle vector useful for transferring heterologous DNA to *P. ruminicola* by conjugation. The shuttle vector comprises: a mobilization region which permits transfer of the shuttle vector from *E. coli* to a colonic Bacteroides species; a mobilization region which permits transfer of the shuttle vector from the colonic Bacteroides species to a *P. ruminicola;* and heterologous DNA operatively linked to a promoter functional in the *P. ruminicola.* These shuttle vectors are particularly advantageous because they can be amplified and manipulated in *E. coli* before they are used to introduce heterologous DNA into *P. ruminicola.*

The invention further provides a tetracycline resistance gene of the TetQ class, or fragments thereof, that confer tetracycline resistance. The TetQ class is a new class of tetracycline resistance genes which confers tetracycline resistance by ribosome protection. The complete DNA sequence of one such gene has been determined and is presented below. The invention also comprises proteins of the TetQ class, or active fragments thereof, that provide tetracycline resistance by ribosome protection.

Finally, the invention provides a promoter functional in *P. ruminicola* and an engineered *P. ruminicola* comprising expressible foreign DNA.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**: A map of pVAL1 and a schematic diagram of the construction of pRDB3, pRDB5 and pNFD13-2. A partial map of the Tc^{r}Em^r DOT element is indicated at the bottom of the figure, and the regions of this element which were cloned into the vectors are indicated by brackets under the map. Abbreviations for restriction sites are: R, EcoRI and C, ClaI. Only relevant restriction sites are shown: Ap^r=ampicillin resistance; Tc^r=tetracycline resistance; Em^r=erythromycin resistance.

FIG. **2A**: Total DNA from the *B. uniformis* 1108 donor containing pRDB5 (lane **1**), and four *P. ruminicola* B$_1$4Tc^{r}transconjugants (lanes **2–5**) was digested with EcoRI. The Southern blot was probed with pFD160 which cross-hydridizes with pRDB5 but not with *P. ruminicola* B$_1$4 DNA.

FIG. **2B**: Total DNA from the *P. ruminicola* B$_1$4R recipient (lane **2**), the *B. uniformis* 1108 donor (lane **3**) and one of the *P. ruminicola* B$_1$4 transconjugants (lane **4**) was digested with EcoRI and HindIII. The Southern blot was probed with XBU4422::pEG920. This probe hybridizes not only with pBR328 sequences on pRBD5 but also with the Tc^{r}Em^r 12256 element in the donor. The two bands corresponding to pRDB5 are indicated in lane **3** by arrows. Lane **1** contains DNA size stan-

dards. The largest four standards are 23.1 kb, 9.4 kb, 6.7 kb and 4.4 kb, respectively.

FIG. 3: Results of pulse field electrophoresis to verify the identity of *P. ruminicola* B_14 Tc^r transconjugants. NotI digests of DNA from the donor, *B. uniformis* 1108 carrying pRDB5, are shown in lanes 1 and 5. NotI digested DNA from *P. ruminicola* GA33 (lane 2), B_14R (lane 3) and one of the B_14 pRDB5 transconjugants (lane 4) are also shown. The NotI pattern of *P. ruminicola* B_14 is identical to that of B_14R (data not shown). The migration distances of some of the yeast chromosomes size standards are shown in megabases (Mb) at the side of the gel.

FIG. 4 is a partial restriction map of the 2.7 kbp SstI clone of the Tc^r gene from *B. thetaiotaomicron* DOT. Important deletion derivatives are indicated by the labeled brackets. The orientation and extent of the large open reading frame encoding TetQ are indicated below by the arrow.

FIGS. 5A and 5B show products of the 2.7 kbp SstI clone in *E. coli*. FIG. 5A is an autoradiogram of a polyacrylamide SDS gel of in vitro transcription. translation products. Lane 1 contains products from the vector control, pFD160. Shown also are products from Tc^s deletion derivatives pNFD13-2ΔRV (lane 2) and pNFD13-2Δ5 (lane 3), reduced Tc^r deletion derivative pNFD13-2Δ4 (lane 4), Tc^r deletion derivative pNFD13-2Δ3 (lane 5) and pNFD13-2Δ1 (lane 6), and intact pNFD13-2 (lane 7). The arrows in the right margin mark the two bands that were consistently unique to the SstI clone in maxicells. FIG. 5B shows an autoradiogram of soluble and membrane fractions from maxicells containing pNFD13-2: Lane 1, soluble fraction; Lane 2, membrane fraction.

FIGS. 6A and 6B show the deduced amino acid sequence of TetQ aligned with representatives of TetO (*Campylobacter jejuni*) and TetM (*Streptococcus faecalis*). The consensus of the sequenced ribosomal protection Tc^r genes is displayed below these sequences. Upper case denotes conservation among the ribosome protection Tc^r proteins. The four barred regions are regions of conservation in GTP-binding proteins [Halliday, *J. Nucleotide Prot. Phosphoryl. Res.*, 9:435–448 (1984)]. Positions marked (*) were found to be involved directly in GTP binding and are invariant in all GTP-binding proteins [Jurnak, *Science.* 230:32–36 (1985)].

FIGS. 7A and 7B show the upstream sequence of tetQ. The endpoints of the pNFD13-2 deletions shown in FIG. 4 are indicated by numbers above the sequence. Only the last three characters of the deletion designations are given. The first letter in parenthesis at each deletion denotes Tc^r expression in *E. coli* (R=resistant; I=intermediate; S=sensitive). The letter following the slash denotes Tc^r expression in Bacteroides. The *E. coli* consensus −35 and −10 sequences are indicated by lines above the tetQ sequence. Below the tetQ upstream sequence is shown the upstream consensus of the tetM sequences from *Staphylococcus aureus, Streptococcus faecalis,* and *Ureaplasma urealyticum* and the tetO sequences from *Campylobacter coli, Campylobacter jejuni,* and *Streptococcus mutans.* Upper case letters denote bases that are conserved in all tetM and tetO sequences. Lower case letters denote bases that are not conserved in all cases, but are the consensus for that position. If data were not available for all six upstream sequences at a position, a lower case letter was used at that position.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

The method of the present invention involves conjugal transfer of shuttle vectors to *Prevotella ruminicola.* As explained in the Background section, *Prevotella ruminicola* are strains of bacteria previously classified as *Bacteroides ruminicola.* The criteria for determining whether a bacterium should be classified as *B. ruminicola* have been loose in the past. Examples of very authentic *B. ruminicola* (now *P. ruminicola*) strains having characteristics quite different than the colonic Bacteroides are B_14, GA33, 23 and 118B. The degree of homology of 16S ribosomal RNA will probably be used as the standard to classify Bacteroides and Prevotella in the near future. Based on this standard, it is expected that B_14, GA33, 23, 118B and bacteria whose 16S ribosomal RNA is at least 70% homologous with that of these strains will be classified as *P. ruminicola.*

The first step of the method of the invention is to transform an *E. coli* with a shuttle vector. Methods of transforming *E. coli* are well known in the art. Any strain of *E. coli* may be used, and numerous strains of *E. coli* are publicly available from such public depositories as the American Type Culture Collection (ATCC). The *E. coli* must have, or be engineered to have, a mobilization element which functions to transfer the shuttle vector from *E. coli* to a recipient colonic Bacteroides species. These elements may be introduced into *E. coli* using methods known in the art. Preferably, the mobilization element is an IncP plasmid, and most preferably the IncP plasmid R751. IncP plasmids such as R751 may be introduced into the *E. coli* by conjugation methods known in the art. Alternatively, *E. coli* strains, such as S17-1, are available which have the IncP plasmid inserted in their chromosomes.

Next, the *E. coli* is contacted with a species of colonic Bacteroides under conditions sufficient so that the shuttle vector is transferred from the *E. coli* to the colonic Bacteroides species. Methods of mating *E. coli* and colonic Bacteroides are known and include those described in Shoemaker, et al., *J. Bacteriol.*, 166:959–965 (1986) and Thomson and Flint, *FEMS Microbiol. Letters,* 61: 101–104 (1989).

Any species of colonic Bacteroides may be used, and many species are available from public depositories, including the ATCC and the Virginia Polytechnic Institute (VPI) Anaerobe Collection (Blacksburg, Va.). The colonic Bacteroides species must contain, or be engineered to contain, a mobilization element which functions to transfer the shuttle vector from the colonic Bacteroides to *P. ruminicola.* These elements may be introduced into the colonic Bacteroides using methods known in the art such as conjugation. The mobilization element is preferably the conjugal element Tc^rEm^r 12256. The Tc^rEm^r 12256 element comprises approximately 120 kb of additional DNA not found in other Bacteroides conjugal elements. Although this segment of DNA has not been fully characterized, it is believed that it may enhance or increase efficiency of transfer. Most preferably, the colonic Bacteroides is *Bacteroides uniformis* containing the Tc^rEm^r 12256 element.

The colonic Bacteroides species is then contacted with a strain of *P. ruminicola* under conditions sufficient so that the shuttle vector is transferred from the colonic Bacteroides species to the *P. ruminicola.* Many suitable species of *P. ruminicola* are available from public depos-

itories, including the ATCC and the VPI Anaerobe Collection. A preferred *P. ruminicola* is B_14.

Since *P. ruminicola* is extremely sensitive to oxygen, conjugation must take place under anaerobic conditions. Further, the use of a modified E (ME) medium has been found critical to obtaining transconjugants. The composition of ME medium is given in Example 1 below.

The present invention also comprises shuttle vectors suitable for transferring heterologous DNA into *P. ruminicola.* A shuttle vector is a vector which contains one or more replicons which allow it to replicate in more than one type of organism. In particular, the shuttle vectors of the present invention must be able to replicate in *E. coli* and colonic Bacteroides. They may also be able to replicate in *P. ruminicola,* or the shuttle vectors, or fragments thereof, may integrate into the *P. ruminicola* chromosome.

Suitable *E. coli* replicons are well-known and include the pUC and pBR series of plasmids. Replicons suitable for use in colonic Bacteroides include pB8-51, pBFTM10, and pBI143 [Salyers, et al., *CRC Critical Reviews in Microbiology,* 14:49–71 (1987): Odelson, et al., *Plasmid,* 17:87–109 (1987); Smith, *J. Bacteriol.,* 164:294–301 (1985)]. It has been found that the pB8-51 replicon also functions in *P. ruminicola.* Other *P. ruminicola* replicons can be identified using the teachings herein and, e.g., the TetQ gene of the invention which is known to be expressed in *P. ruminicola.*

The shuttle vectors of the invention must also be capable of being transferred from *E. coli* to a colonic Bacteroides species. Accordingly, they must contain a mobilization region which permits this transfer. The mobilization region must be one which is acted on by the mobilization element present in the *E. coli* to effect the transfer. Suitable mobilization regions are known. They include those on pBFTM10 (pDP1, pCG30), pB8-51 (pEG920, pVAL1), and pBI143 (pFD160) which are mobilized by IncP plasmids [Salyers, et al., *CRC Critical Reviews in Microbiology,* 14:49–71 (1987); Odelson, et al., *Plasmid,* 17:87–109 (1987); Shoemaker, et al., *J. Bacteriol.,* 166 959–965 (1986)].

The shuttle vectors must also be capable of being transferred from the colonic Bacteroides species to *P. ruminicola,* and they must contain a mobilization region which permits this transfer. The mobilization region must be one which is acted on by the mobilization element present in the colonic Bacteroides to effect the transfer. Suitable mobilization regions include the mobilization region of pB8-51 which is mobilized by Tc^rEm^r 12256. Other mobilization regions can be identified using the teachings herein.

The shuttle vector also comprises heterologous DNA sought to be transferred to *P. ruminicola.* "Heterologous DNA" is defined herein to mean DNA from a source other than the *P. ruminicola* strain which is to receive the heterologous DNA. The heterologous DNA may include DNA encoding enzymes involved in the fermentation of carbohydrates in the rumen, enzymes involved in the degradation of polysaccharides (such as xylanase or polysaccharases), other enzymes involved in rumen metabolism, and enzymes or groups of enzymes that synthesize substances that are beneficial to growth of cattle such as antibiotics. By transferring heterologous DNA to *P. ruminicola,* new and useful traits may be imparted to the recipient *P. ruminicola.* These traits can include those which will lead to more economical beef production.

The heterologous DNA is operatively linked to a promoter functional in *P. ruminicola.* A preferred promoter is a promoter of a TetQ gene (see discussion of TetQ genes below). Another preferred promoter is from the Tc^rEm^r DOT element. A particularly preferred promoter comprises the sequence (SEW ID NO: 1)

```
AAAAATCCTC CTACTTTTGT TAGATATATT TTTTTGTGTA ATTTTGTAAT   50
CGTTATGCGG CAGTAATAAT ATACATATTA ATACGAGTTA TTAATCCTGT  100
AGTTCTCATA TGCTACGAGG AGGTATTAAA AGGTGCGTTT CGACAATGCA  150
TCTATTGTAG TATATTATTG CTTAATCCAA,                       180
```

or active variants thereof. This promoter is the promoter region of the Tc^r gene of the Tc^rEm^r element of *B. thetaiotaomicron* DOT and may be isolated from that gene or may be prepared by chemical synthesis. This promoter region is also strongly believed to be sufficient to initiate transcription in *P. ruminicola.* "Active variants" are promoters which have deletions, additions and/or substitutions of nucleotides as compared to the above sequence, but which are still able to initiate transcription in *P. ruminicola.*

The shuttle vector will also include one or more selection markers. Selection markers must be used to distinguish transformed *E. coli* from untransformed *E. coli* and to distinguish transconjugant colonic Bacteroides and *P. ruminicola* from non-transconjugants. It is also necessary to include selection markers that distinguish donor from recipient in mating mixtures. Many suitable selection markers are known and include antibiotic resistance, amino acid or other nutrient requirements, pH, and combinations of these. Preferred selection markers for *P. ruminicola* are TetQ tetracycline resistance genes. Especially preferred is the TetQ tetracycline resistance gene isolated from the Tc^rEnm^r-DOT element whose sequence is given below.

The various components of the shuttle vector may be isolated or synthesized and then assembled using techniques that are well known in the art. Indeed, one the most important aspects of the present invention is that it allows for the engineering of DNA that is to be introduced into *P. ruminicola.*

A preferred shuttle vector is pRDB5. The chimeric pRDB5 construct contains sequences from the plasmid pBR328, a cryptic colonic Bacteroides plasmid, pB8-51, and a colonic Bacteroides Tc^r gene isolated from the Tc^rEm^r-DOT conjugal element. The restriction map of pRDB5 is shown in FIG. 1. Plasmid pRDB5 replicates in *E, coli,* colonic Bacteroides and *P. rumininicola.* Although it is not known whether pRDB5 replicates in, or transfers to, all colonic Bacteroides and *P. ruminicola,* this plasmid has a broad host range, and it is likely it can be used in many colonic Bacteroides and *P. ruminicola.*

In a preferred embodiment of the method of the present invention, *E. coli* were transformed with pRDB5. Then pRDB5 was mobilized from *E. coli* into *B. uniformis* by the IncP plasmid R751 which was present in the *E. coli.* Next, pRDB5 was conjugally transferred from *B. uniformis* to *P. ruminicola* B_14 by the conjugal element Tc^rEm^r 12256 present in the *B. uniformis.* A combination of in vitro sections was utilized to identify *P.*

ruminicola B_14 transconjugants. First, the *P. ruminicola* B_14 recipient used was a rifampicin resistant mutant (rif^r) produced by growing *P. ruminicola* B_14 on increasing levels of rifampicin to produce a spontaneous mutant. The rif^r *P. ruminicola* B_14 transconjugants could then be selected against donor *B. uniformis*, a species that is rifampicin sensitive. *B. uniformis* 1100 was chosen as a donor because it is a thymidine auxotroph, and the lack of thymidine in the selection medium could be used to select against that donor after matings with *P. ruminicola* B_14. *B. uniformis* is also known to grow in medium containing vitamin K, whereas *P. ruminicola* B_14 has no vitamin K requirement. Thus, vitamin K was also omitted from the selection medium. Finally, pH was used in the selection method because *P. ruminicola* B_14 grows well at pH 6.2, whereas *B. uniformis* does not grow well at pH values lower than 6.8. The combination of selection for antibiotic resistance, lack of thymidine and vitamin K, and low pH provided a relatively clean background for selecting *P. ruminicola* B_14 transconjugants. The transconjugants were distinguished from non-transconjugant *P. ruminicola* B_14 because they were tetracycline resistant due to the expression of the foreign Tc^r gene on pRDB5. The *P. ruminicola* B_14 transconjugants were also tested for other traits that characterize *P. ruminicola* B_14 and differentiate that strain from the donor *B. uniformis*. The results of the tests demonstrated that true *P. ruminicola* transconjugants containing pRDB5 were produced by the method of the invention.

The present invention also comprises transconjugant *P. ruminicola* prepared by the method of the invention and containing the shuttle vectors of the invention A particularly preferred transconjugant is *P. ruminicola* B_14 containing pRDB5.

The invention further comprises a tetracycline resistance gene of the TetQ class, or fragments thereof that confer tetracycline resistance. The TetQ class is a new class of tetracycline resistance genes which confers tetracycline resistance by coding for proteins which protect ribosomes from the inhibitory binding of tetracyline.

The invention also comprises the proteins encoded by the TetQ genes (hereinafter "TetQ class of proteins"), or active fragments thereof. "Active fragments" of these proteins are fragments which are still capable of conferring tetracycline resistance. The DNA sequence of one TetQ gene (isolated from the Bacteroides conjugal element Tc^rEm^r-DOT) has been determined and is presented below in Example 2, along with the amino acid sequence of the protein encoded by the gene. The invention also comprises other DNA sequences which encode this same protein.

Hybridization studies using a portion of the sequenced gene indicates that TetQ genes are widespread in colonic Bacteroides. Given the stringency used in these experiments, it is estimated that the Tc^r genes found in other Bacteroides Tc^r strains share at least 80% identity with the sequenced gene. Also, the Tc^r gene on the *P. ruminicola* plasmid pRRI4 appears to be a TetQ gene.

TetQ genes may be isolated from Bacteroides and Prevotella Tc^r strains using known techniques. Alternatively, genes, or gene fragments, may be prepared using chemical synthesis.

Finally, the invention provides an engineered *P. ruminicola* containing expressible foreign DNA. "Foreign DNA" is used herein to mean DNA from a source other than *P. ruminicola*. Thus, "foreign DNA" is more narrow than "heterologous DNA," and heterologous DNA includes foreign DNA. "Engineered" is used to mean *P. ruminicola* not found in nature.

EXAMPLES

The restriction enzymes used in the following examples were obtained from Bethesda Research Laboratory, Gaithersburg, Md. They were used according to the manufacturer's instructions.

EXAMPLE 1

A. A Construction Of Shuttle Vectors

Four shuttle vectors were constructed. They were pRDB5, pVAL1, pRDB3, and pNFD13-2, shown in FIG. 1.

The vector pVAL1 carries the erythromycin resistance (Em^r) gene from the colonic Bacteroides transposon Tn4351 linked to portions of pBR328 (an *E. coli* replicon) and the cryptic Bacteroides plasmid pB8-51 (a colonic Bacteroides replicon). It was prepared as described in Valentine, et al., *J. Bacteriol.*, 170:1319–1324 (1988). Briefly, pBR328 (available from Boehringer Mannheim) was digested with EcoRI. The EcoRI fragment of Tn4351 [preparation from pBF4 described in Shoemaker, et al., *J. Bacteriol.*, 162:626.632 (1985)] was ligated to the EcoRI-digested pBR328 to produce pTB1. Plasmid pB8-51 was isolated from *Bacteroides eggerthi* by standard plasmid isolation techniques [See Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor, N.Y. 1982)]. It was then partially digested with TaqI. Next, ClaI digests of pTB1 were mixed with the TaqI digests of pB8-51 and ligated with T4 DNA ligase to produce pVAL1.

Vector pRDB3 was prepared by cloning a 7 kbp HincII fragment from a cosmid clone of the Tc^rEm^r-DOT element into pVAL1. The cosmid clone was prepared as described in Shoemaker, et al., *J. Bacteriol.*, 171:1294–1302 (1989). Then the cosmid clone was digested with HincII, and the resulting 7 kbp fragment containing the Tc^r gene was ligated to PvuII digested pVAL1 to produce pRDB3.

Next, pRDB3 was digested with ClaI and religated to produce pRDB5. The result of this manipulation was to remove the Tn4351 Em^r gene.

The vector pNFD13-2 comprises pFD160 having a 2.7 kbp fragment containing the Tc^r gene from Tc^rEm^r-DOT cloned into the SstI site. Plasmid pFD160 was prepared as described in Smith, *J. Bacteriol.*, 164:294–301 (1985). It consists of HaeII-cleaved pBI143 (a colonic Bacteroides replicon) ligated to NdeI-digested pUC19 (an *E. coli* replicon). The 2.7 kbp fragment containing the Tc^r gene was prepared as follows. Tn1000 insertions into pRDB3 were used to create convenient restriction sites. Transposon mutagenesis was performed by transforming an *E. coli* strain carrying the F plasmid on which Tn1000 resides with pRDB3. Tn1000 causes cointegrates to form between pRDB3 and the F plasmid. During conjugation, F::pRDB3 cointegrates are transferred to a recipient. In the recipient, the cointegrates resolve, leaving the F plasmid and pRDB3 with a Tn1000 insertion.

Restriction digests of the resulting pRDB3::Tn1000 isolates were screened by standard techniques (Maniatis, et al., supra), and the smallest clone that would express Tc^r in colonic Bacteroides was identified. This

clone was the 2.7 kbp fragment containing the Tc^r gene and was excised with SstI.

B. Transformation Of *E. coli*

E. coli donor strains were constructed by introducing pRDB5, pVAL1, or pNFD13.2 into *E. coli* DH5αMCR [obtained from Bethesda Research Laboratory] or S17-1 [obtained from R. Simon, Universitat Bielefeld, Postfach 86–40, D-4800 Bielefeld 1, FRG; described in Simon, et al., Bio/Technology, 1:784.791 (1983)]. The plasmids were introduced into the *E. coli* strains by transformation techniques previously described [See Maniatis, et al., supra]. The IncP mobilizing plasmid R751 [See Meyer, et al., J. Bacteriol., 143:1362–1373 (1980)] was introduced into *E. coli* DH5αMCR by conjugation as described in Shoemaker, et al., *J. Bacteriol.*, 171:1294–1302 (1989); Thomson, et al., *FEMS Microbiol. Letters*, 61:101–104 (1989). *E. coli* S17-1 had a copy of the IncP plasmid RP4 already inserted in its chromosome. Both R751 and RP4 mobilize pRDB5, pVAL1, and pNFD13-2 from *E. coli* to *B. uniformis* at frequencies of 10^{-4} per recipient.

C. Preparation Of Colonic Bacteroides Donors

B. uniformis donor strains containing Tc^{r}Em^r element 12256 and pRDB5 (Tc^r) or pNFD13-2 (Tc^r) were constructed by first introducing the plasmid pRDB5 or pNFD13-2 into *B. uniformis* 1100 [obtained from the VPI Anaerobe Laboratory, Blacksburg, Va.], as described previously [Shoemaker, et al., *J. Bacteriol.*, 166:959–965 (1986); Thomson, et al., *FEMS Microbiol. Letters*, 61:101–104 (1989)], and selecting for tetracycline resistance. Transconjugants carrying the Tc^r plasmid were used as recipients in a mating with *B. uniformis* 1008 (Tc^{r}Em^r) obtained from the VPI Anaerobe Laboratory] to transfer the Tc^{r}Em^r element 12256, with selection for Tc^r and Em^r. The resulting strains were designated *B. uniformis* 1108 (pRDB5) and *B. uniformis* 1108 (pNFD13-2).

Similarly, to construct *B. uniformis* carrying the Tc^{r}Em^r element 12256 and pVAL1 (Em^r), pVAL1 was first transferred from *E. coli* to *B. uniformis* 1100 by conjugation, with selection for Em^r. Then, the Tc^{r}Em^r 12256 element was introduced by conjugation from *B. uniformis* 1008 to *B. uniformis* 1100 (pVAL1), with selection for Tc^r and Em^r. The final strain was designated *B. uniformis* 1108 (pVAL1).

D. Mating with *P. ruminicola*

Next, the recipient, *P. ruminicola* B_14 (obtained from Marvin Bryant, Dept. of Animal Sciences, University of Illinois, Urbana, Ill.), was mated with *E. coli* or *B. uniformis*. *E. coli* donor strains were grown in Luria broth (LB) to an O.D. (650 nm) of 0.15–0.20. *B. uniformis* 1108 strains were grown in TYG-Thy-K broth in 80% nitrogen-20% carbon dioxide to an O.D. (650 nm) of 0.15–0.20. Optical densities were measured in 18 mm diameter culture tubes in a Spectronic 20 spectrophotometer (Milton Roy Co., Rochester, N.Y.). TYG-Thy-K broth is trypticase-yeast extract-glucose broth [composition given in Holdeman, et al., *Anaerobe Laboratory Manual* (4th ed., Virginia Polytechnic Institute, Blacksburg, Va. 1977)] containing 100 μg/ml thymidine and 1 μg/ml vitamin K_3, with a final pH of 7.0–7.3.

P. ruminicola B_14 was grown in MM10 broth at 80% nitrogen-20% carbon dioxide to an O.D. (650 nm) of 0.25–0.30. MM10 is similar to M10 medium previously described [*Anaerobe Laboratory Manual*, supra], except the concentration of trypticase and yeast extract was increased ten-fold and amylopectin was present as the carbohydrate source. Also, titanium citrate (0.15M) was added drop-wise until the resazurin became colorless (approximately 0.2–0.3 ml per liter of medium) prior to the addition of cysteine. The pH of this medium was 6.5–6.6. This medium, as were all media used for culturing *P. ruminicola*, was made in glass tubes sealed with a rubber stopper.

The *E. coli* or *B. uniformis* donor (30 ml) was centrifuged in a Sorvall GLC28 bench top centrifuge (SP/X rotor; Dupont Instruments, Wilmington, Del.) at 3,000 rpm for 15 minutes at room temperature to pellet the bacteria. The bacteria were then washed in 5 ml potassium phosphate buffer (0.1M, pH 7.0) and resuspended in 1 ml of TYG-Thy-K medium. Manipulations of *E. coli* or *B. uniformis* were performed under aerobic conditions.

P. ruminicola B_14 (10 ml) was centrifuged in sealed culture tubes at 3,000 rpm for 15 minutes at room temperature as described above, and the supernatant fluid was removed with a sterile syringe.

The resuspended donor (*E. coli* or *B. uniformis*) (1 ml) and 5 ml of anaerobic 0.1M potassium phosphate buffer (pH 7.0) were injected into the tube. Anaerobic phosphate buffer was prepared by boiling phosphate buffer and cooling under a stream of oxygen-free carbon dioxide. After vortexing the tubes to dislodge the pelleted recipient, the bacterial mixture was centrifuged again in the sealed tubes, and the wash solution was withdrawn with a syringe. TYG-Thy-K medium (1.5 ml) and MM10 medium (1.5 ml) were injected into the tube, and the tube was vortexed to resuspend the bacteria. The resuspended mixture was injected into a sealed anaerobic tube containing a slant of modified E agar medium ("ME"), pH 6.8, for the mating. ME is the same as Sweet E medium previously described (*Anaerobe Laboratory Manual*, supra), except it contains glucose, as the only carbohydrate, and 100 ug/ml thymidine. Agar was added to a final concentration of 2%. The tubes were then centrifuged as described above to pellet the bacteria on the slants. The tubes were inverted gently, and the supernatant fluid removed with a syringe. The tubes were then incubated upside down at 37° C. for 15–18 hours.

After incubation, 1 ml of MM10 (pH 6.6), containing no thymidine or vitamin K was added to the slant tubes, and the tubes were vortexed. Next, resuspended bacteria were removed with a sterile syringe which had been gassed out with nitrogen-carbon dioxide.

To select for transconjugants, 0.1–0.2 ml of resuspended cells, or 0.1–0.2 ml of a 1:10 dilution, were inoculated into a roll tube containing MM10-Rif-Tc or MM10-Rif-Em selection medium. MM10-Rif (pH 6.2) medium consisted of MM10 containing 2% agar and 40 ug/ml rifampicin. For selection of transconjugants, either tetracycline (final concentration of 5 ug/ml) or erythromycin (final concentration of 5 ug/ml) was added to the MM10-Rif medium to produce MM10-Rif-Tc and MM10-Rif-Em, respectively.

To enumerate the total number of *B. uniformis* donors, 0.1 ml of a 10^{-6} dilution of the resuspended cells was plated on TYG-Thy-K agar plates and incubated in a GasPak jar. To enumerate the *E. coli* donors, 0.1 ml of a 10^{-6} dilution was plated on LB agar and incubated aerobically. To enumerate the *P. ruminicola* B_14 recipients, 0.1 ml of a 10^{-6} and a 10^{-8} dilution were inocu-

lated into an MM10-Rif roll tube. All incubations were done at 37° C. for 3.4 days.

E. Results Of *E. coli-P. ruminicola* Matings

When the transfer of plasmids pVAL1, pNFD13-2, and pRDB5 from *E. coli* to *P. ruminicola* was attempted, no Tc^r or Em^r *P. ruminicola* transconjugants were detected. As a result, *B. uniformis* was used as an intermediate donor for *P. ruminicola* as described in the next section.

F. Results Of *B. uniformis-E. coli* Matings

B. uniformis 1108 (pRDB5), *B. uniformis* 1108 (pVAL1), or *B. uniformis* 1108 (pNFD13-2), prepared as described above, were mated with *E. coli* HB101 or EM24 to determine whether the *B. uniformis* recipients carrying a conjugal Tc^rEm^r 12256 element and a plasmid were capable of mobilizing the plasmid at high frequency. The procedure for the *B. uniformis-E. coli* mating has been described previously [See Shoemaker, et al., *J. Bacteriol.*, 166:959–965 (1986); Thomson, et al., *FEMS Microbiol. Letters*, 61:101–104 (1989)]. Mobilization of these plasmids from *B. uniformis* to *E. coli* occurred at frequencies of 10^{-4}–10^{-5} per recipient (see Table 1 below).

G. Results Of *B. uniformis-P. ruminicola* Matings

To test for transfer of the plasmids from *B. uniformis* to *P. ruminicola* B_14, a selective medium allowing growth of *P. ruminicola* but not *B. uniformis* had to be developed. Being able to detect transfer frequencies as low as 10^{-9} per recipient was the criterion.

First, the antibiotic sensitivity of *P. ruminicola* B_14 was tested. Minimal inhibitory concentrations for various antibiotics were determined by inoculating MM10 containing different concentrations of antibiotic and incubating for 48 hours. Antibiotic concentrations tested were 5, 10, 20, 50, 100 and 200 ug/ml. In the case of tetracycline and erythromycin, resistance levels on MM10 agar medium were also determined.

P. ruminicola B_14 was found susceptible to rifampicin (10 ug/ml), tetracycline (2 ug/ml), erythromycin (1 ug/ml), gentamicin (20 ug/ml), and ampicillin (5 ug/ml). It was resistant to chloramphenicol (10 ug/ml), kanamycin (50 ug/ml), trimethoprim (200 ug/ml), and nalidixic acid (100 ug/ml).

P. ruminicola B_14 was susceptible to all of the antibiotics which inhibited growth of *B. uniformis* except chloramphenicol. Accordingly, chloramphenicol was first used to select for *P. ruminicola* B_14 and against the donor. Mixtures of *B. uniformis* and *P. ruminicola* B_14 were plated on MM10 agar containing 10 ug/ml chloramphenicol. Donor *B. uniformis* colonies were still able to grow enough to obscure true transconjugants. Therefore, another resistance for selecting *P. ruminicola* recipients was required.

A spontaneous rifampicin resistant (Rif^r) mutant of *P. ruminicola* B_14 was isolated by inoculating the bacteria into MM10 broth medium containing progressively higher concentrations of rifampicin. By growing *P. ruminicola* B14 on successively higher concentrations, a spontaneous mutant of *P. ruminicola* B_14 was obtained which would grow in rifampicin concentrations as high as 60 ug/ml. The spontaneous Rif^r mutant was determined to be a derivative of *P. ruminicola* B_14 by comparing its NotI digest pattern with that of the original B_14 strain. The restriction enzyme digest patterns were identical. This Rif^r strain was used in matings to provide a selection for the *P. ruminicola*. This method of producing the rifampicin mutant is a well known method of producing suitable *P. ruminicola* rifampicin resistant mutants can be produced in this manner.

However, using the Rif^r derivative, *P. ruminicola* B_14R, as a recipient and selecting for rifampicin resistance did not allow for the detection of transfer frequencies as low as 10^{-9} per recipient because spontaneous Rif^r mutants of *B. uniformis* 1108 occurred at a frequency of 10^{-7}.

Accordingly, a combination of selections had to be used. First, the *P. ruminicola* B_14 rifampicin resistant mutant was used. *B. uniformis* 1100 was chosen as a donor because it is a thymidine auxotroph, and the lack of thymidine in the selection medium could be used to select against that donor after matings with *P. ruminicola* B_14. However, spontaneous reversion to wild type occurs at relatively high frequencies (10^{-6}). *B. uniformis* is also known to grow in medium containing vitamin K, whereas *P. ruminicola* B_14 has no vitamin K requirement. Thus, vitamin K was also omitted from the selection medium. Finally, pH was used in the selection method because *P. ruminicola* B_14 grows well at pH 6.2, whereas *B. uniformis* does not grow well at pH values lower than 6.8. The combination of selection for antibiotic resistance, lack of thymidine and vitamin K, and low pH provided a relatively clean background for selecting *P. ruminicola* B_14 transconjugants.

Using this selection medium and using a donor to recipient ratio of 1.5–3.0:1.0, Tc^r transconjugants were detected in a mating between *B. uniformis* 1108 (pRDB5) and *P. ruminicola* B_14 (Rif^r) at frequencies of 10^{-6}–10^{-7} per recipient (see Table 1). No transconjugants were detected in matings in which the donor was *B. uniformis* 1108 (pNFD13-2) or *B. uniformis* 1108 (pVAL1).

The ability of the transconjugants to grow in various media was tested to rule out the possibility that the apparent transconjugants were spontaneous Rif^r or Rif^rThy^+ mutants of the *B. uniformis* donor. Growth on TYG, no growth on TYG-Thy, no growth in MM10 containing gentamicin, and growth in MM10 containing xylan instead of glucose was observed. These phenotypic characteristics indicated that the transconjugants were of *P. ruminicola* origin rather than *B. uniformis*.

DNA analysis of *P. ruminicola* B_14 transconjugants was performed. Plasmids were isolated from *P. ruminicola* B_14 transconjugants by the Ish-Horowitz modification of the Birnbom and Doly procedure as described in Maniatis, et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1982). Southern blots were also performed as described in Maniatis, et al., supra. Total DNA was prepared by standard methods as described in Saito, et al., *Biochem. Biophys. Acta.* 72:619–629 (1963); Shoemaker, et al., *J. Bacteriol.*, 171:1294–1302 (1989); Shoemaker, et al., *J. Bacteriol.*, 166:959–965 (1986).

Plasmid preparations made from *P. ruminicola* B_14R had a background staining material that made it difficult to see plasmid DNA unambigiously. However, when a plasmid preparation was used to transform *E. coli* and pRDB5 was recovered in *E. coli*, the restriction profile of this plasmid was identical to that of the original pRDB5.

Additionally, total DNA (plasmid plus chromosome) was isolated from apparent *P. ruminicola* transconjugants, digested with EcoRI and subjected to Southern

blot analysis. EcoRI cuts once in pRDB5 to produce a 15 kb linear segment. The DNA digests were separated on a 1.0% agarose gel and blotted onto Optibind (Schleicher and Schuell). The digests were then probed with ^{32}P-labelled pFD160. The pFD160 plasmid hybridizes with the pBR328 sequences in pRDB5, but not with the Tc^r gene. All of the putative transconjugants contained a single band of the correct size which hybridized with the probe (see FIG. 2A).

Total DNA from *B. uniformis, P. ruminicola* B_14, and *P. ruminicola* transconjugants were also digested with HindIII and EcoRI restriction enzymes, and the digests blotted onto Optibind. The blot was hybridized with labelled XBU4422::pEG920 [prepared as described in Shoemaker, et al., *J. Bacteriol.*, 172:1694–1702 (1990)], a probe which detects pRDB5 and the TcEm^r 12256 element. If the apparent transconjugants were Thy$^+$-Rif^r mutants of *B. uniformis* 1108 (pRDB5), the Southern blot would show a number of bands, including the two bands produced from a HindIII-EcoRI digest of pRDB5. As can be seen from FIG. 2B, a mixture of bands due to pRDB5 and the Tc^{r}Em^r 12256 element was seen in the *B. uniformis* donor, whereas only the bands associated with pRDB5 were seen in the transconjugant. These results indicated that the transconjugants were not revertants of the *B. uniformis* donor.

The NotI restriction enzyme digest patterns of DNA from *B. uniformis, P. ruminicola* B_14R, and a *P. ruminicola* B_14 transconjugant were compared on pulsed field gels to determine whether a Tc^r contaminant having properties similar to *P. ruminicola* had been isolated instead of true transconjugants. The NotI digest pattern of *P. ruminicola* B_14 differs not only from that of *B. uniformis* 1108, but also differs from that of other *P. ruminicola* strains (data not shown). As shown in FIG. 3, the NotI restriction patterns of the *P. ruminicola* B_14R recipient and the Tc^r transconjugant were identical to each other and to that of *P. ruminicola* B_14.

The combined data show that true *P. ruminicola* transconjugants were obtained.

No transfer of pNFD13-2 to *P. ruminicola* B_14 was detected. Since pNFD13-2 has the same Tc^r gene as pRDB5 but derives its replication region from a different plasmid, the lack of transconjugants was most likely due to failure of the pNFD13-2 replication origin (pBI143) to work in *P. ruminicola* B_14. However, there is a 4 kbp region upstream of the Tc^r gene which is present in pRDB5 but not in pNFD13-2. This region seems to have no effect expression of the Tc^r gene in *B. uniformis,* but it might affect expression in *P. ruminicola* B_14. Since pNFD13-2 was mobilized from *B. uniformis* to *E. coli* at frequencies comparable to mobilization frequencies seen with pRDB5, it is possible that pNFD13-2 is getting into *P. ruminicola* B_14 but is lost because it cannot replicate. If so, pNFD13-2 could serve as a suicide vector for introducing DNA into the chromosome of *P. ruminicola* B_14.

Genetic manipulation of *P. ruminicola* would be easier if *E. coli* were the donor. Failure to demonstrate transfer of pRDB5 from *E. coli* to *P. ruminicola* could be due to the failure of IncP plasmids to mediate formation of mating pairs between *E. coli* and *P. ruminicola.* However, since IncP plasmids mediate mating between *E. coli* and the colonic Bacteroides strains, this seems unlikely. A more likely possibility is that the transfer frequency is lowered by the anaerobic mating conditions. Aerobic matings with the oxygen-sensitive *P. ruminicola* are not feasible. Nonetheless, it may be possible to find conditions that raise the frequency of mating and allow *P. ruminicola* to survive. Finally, restriction enzymes in *P. ruminicola* may prevent survival of pRDB5 introduced from *E. coli.*

TABLE 1

Transfer frequencies of various shuttle vectors from *B. uniformis* to either *B. ruminocola* B_14 or *E. coli.*

Donor strain	Frequency of transfer from *B. uniformis* to *E. coli*	Frequency of transfer from *B. uniformis* to *B. ruminicola*
B. uniformis 1108 (pRDB5)	3×10^{-4}[a]	$10^{-7} - 10^{-6}$
B. uniformis 1108 (pNFD13-2)	1×10^{-4}	$<10^{-9}$
B. uniformis 1108 (pVAL1)	1×10^{-4}	$<10^{-9}$
B. uniformis 1104 (pRBD3)	1×10^{-5}	$<10^{-9}$
B. uniformis 1108 (pRDB5-2)[b]	2×10^{-4}	$10^{-7} - 10^{-6}$

[a]Frequencies are given as transconjugants per recipient. Numbers represent the mean of at least three separate experiments.
[b]pRDB5-2 is pRDB5 isolated from a *B. ruminicola* B_14 transconjugant in the *B. uniformis* 1108 background.

EXAMPLE 2

The Bacteroides Tc^r gene, originally derived from the Bacteroides conjugal element Tc^{r}Em^r-DOT [Shoemaker, et al., *J. Bacteriol.*, 171:1294–1302 (1989)], was subcloned on a 2.7 kbp fragment, and the 2.7 kbp fragment was sequenced. The complete sequence of the fragment is shown below in Chart A. Computer analysis of the DNA sequence, translation into amino acid sequence, and comparisons to amino acid sequences of other tetracycline resistance peptides were performed. The amino acid sequence of the gene product is presented below in Chart B. A promoter region functional n Bacteroides species was identified. Its sequence is shown below in Chart C. This promoter region is also strongly believed to be sufficient to initiate transcription in *P. ruminicola.*

The gene coded for a protein of the ribosome protection type of tetracycline resistance. However, the amino acid sequence coded for by the cloned gene was found to be only about 40% identical to sequences coded for by the TetM and TetO genes, two known classes of ribosome protection type tetracycline resistant genes. Accordingly, it was concluded that the Bacteroides Tc^r was clearly in a separate DNA-DNA hybridization class from TetM and TetO and constituted its own DNA hybridization class. This new class of tetracycline resistance genes is designated TetQ. The experiments and analyses performed, and the Bacteroides Tc^r gene and its gene product, will now be described in detail.

A. Materials and Methods

1. Strains and Growth Conditions

Strains used in this study are listed in Table 2. *E. coli* DH5α was obtained from Bethesda Research Laboratory. *B. thetaiotaomicron* strains BT 4001, BT4002, BT4004, BT4007 and BT4008 and *B. uniformis* BU10001 are described in Shoemaker and Salyers, *J. Bacteriol.*, 170:1651–1657 (1988). *B. thetaiotaomicron* strains 5482 and 2808, *B. uniformis* strains C7–17, 2537, T1–1, *B. distasonas* strains 4243, C30-45, 6308, and *B. caccae* strains 3452A and 8608 are described in Johnson, *J. Syst. Bacteriol.*, 28:245–256 (1978). *B. fragilis* AK87 was obtained from A. Kuritza, Yale University Medical School, New Haven, CT. *E. coli* LCD44 was obtained from Dr. John Cronon, Jr., University of Illinois, Urbana, Ill.

Bacteroides strains were grown either in prereduced Trypticase (BBL Microbiology Systems)-yeast extract-glucose (TYG) [Holdeman, et al., *Anaerobe Laboratory Manual,* supra] under an 80% N_2/20% CO_2 atmosphere or on TYG agar plates in a GasPak jar. *E. coli* strains were grown in Luria broth (LB) or on LB agar plates unless otherwise indicated.

2. Plasmids

The preparation of pNFD13-2 is described above in Example 1. As discussed there, it contains a 2.7 kbp insert containing the Tc^r gene of the Tc^rEm^r-DOT element. Plasmid pNFD13-6 is identical to pNFD13-2, but with the 2.7 kbp insert in the opposite orientation.

3. DNA Isolation and Analysis

Plasmids were isolated from *E. coli* by the Ish-Horowitz modification of the Birnboim and Doly method [Maniatis, et al., supra]. Chromosomal DNA from Bacteroides was isolated by the method of Saito and Miura, *Biochim. Biophys. Acta.* 72:619–629 (1963). Restriction digestion and ligation with T4 DNA ligase followed standard procedures (Maniatis, et al., supra). Electrophoretic resolution of restriction digests was done in 0.8–1.0% agarose slab gels in 1X or 4X GGB (1X: 0.04M Tris, 0.02M sodium acetate, 0.002M EDTA). Gels were stained with ethidium bromide (1 μg/ml) and photographed. Plasmids were introduced into *E. coli* employing the transformation procedure of Lederberg and Cohen, *J. Bacteriol.* 119:1072–1074 (1974).

4. Southern Hybridization

For Southern blot hybridization analysis, DNA was digested with restriction enzymes and electrophoresed on a 1% agarose gel. The DNA was transferred to Millipore HAHY nitrocellulose paper by capillary blotting (Maniatis, et al., supra). Nick translation was used to label DNA probes with [α-^{32}P]-dCTP [Rigby, et al., *J. Mol. Biol.,* 113:237–251 (1977)]. Probes were hybridized to DNA on the nitrocellulose paper for 24 hours at 42° C. in a hybridization solution containing 50% formamide (Maniatis, et al., supra). Following hybridization, blots were washed twice for 30 minutes each with 2X SSC (0.3M NaCl and 0.03M sodium citrate) containing 0.2% sodium dodecyl sulfate (SDS), then twice with 0.2% SDS in 0.5X SSC at 60° C. Blots were then analyzed using autoradiography.

5. Minimum Inhibitory Concentration (MIC) of Tetracycline

To test for expression of the 2.7 kbp clone of the Tc^r gene and its various deletion derivatives in *E. coli* and *B. thetaiotaomicron,* MIC values were determined. When *E. coli* was the host, ampicillin (100 μg/ml) or tetracycline (3 μg/ml) was added to inoculum cultures to maintain plasmids in plasmid-bearing strains. In most experiments, MIC values were determined using the tube dilution method. Cells (0.1 ml) from overnight inoculum cultures were introduced into LB broth medium containing serially incremented concentrations of tetracycline. Increments of 5 μg/ml were used. Tubes were incubated at 37° C. and scored visually for growth at 12 and 24 hours. In some experiments, the level of resistance was determined by patching cultures onto LB agar plates containing different concentrations of antibiotic and scoring growth after 24 hours.

To test for expression in Bacteroides, vectors containing various subclones of the 2.7 kbp clone of the Tc^r gene were mobilized into *B. thetaiotaomicron* as described previously [Shoemaker, et al., *J. Bacteriol.,* 171:1294–1302 (1989); Shoemaker, et al., *J. Bacteriol.,* 162:626–632 (1985)], with selection for Tc^r. Transfer frequencies were several logs above background. Thus, failure to obtain a Tc^r transconjugant was a reasonable indication that the deletion clone failed to express Tc^r in Bacteroides. In Bacteroides, MIC determinations were done in TYG broth medium with serially incremented concentrations of tetracycline.

6. Maxicells

The maxicell procedure was executed as described by Sancar, et al., *J. Bacteriol.,* 137:692–693 (1979), with *E. coli* LCD44 as host. Samples were solubilized by incubation in SDS or lithium dodecyl sulfate solubilizing solution at 37° C. to avoid possible aggregation. Proteins from maxicells were separated by electrophoresis on 11% highly cross-linked SDS polyacrylamide gels as described by Hashimoto, et al., *Anal. Biochem.,* 112:192–199 (1983). Following electrophoresis, gels were stained with Fast Stain (Zoion Research Inc., Allston, Mass.), dried onto filter paper under vacuum, and autoradiographed. Molecular weight markers from BRL, Gaithersburg, Md., were used for size estimation. Maxicell fractionation was performed using an adaptation of the method of Tai and Kaplan, *J. Bacteriol.,* 164:83–88 (1985).

7. In Vitro Transcription and Translation

Proteins encoded by plasmid templates were compared using an *E. coli*-derived in vitro transcription-translation system [DeVries and Zubay, *Proc. Nat. Acad. Sci. USA.* 57:1010–1012 (1967)] in kit form (Amersham, Arlington Heights, Ill.). Radiolabeled proteins were resolved on 11% highly cross-linked SDS polyacrylamide and detected by autoradiography as described above.

8. DNA Sequencing and Analysis

The region sequenced in this study was the 2.7 kbp SstI fragment from pNFD13.2 and pNFD13-6. Progressive unidirectional deletions were introduced into the insert DNA using an adaptation of the exonuclease III procedure of Henikoff, *Gene,* 28:351 (1984), provided in kit form (Erase-a-Base System by Promega, Madison, Wisc.). (See FIG. 4) Both strands were sequenced by the dideoxy chain termination reaction with the T7 DNA polymerase variant and reagents provided in the Sequenase 2.0 kit Biochemicals, Cleveland, Ohio). Computer analysis of DNA sequence, translation into amino acid sequence and comparisons to amino acid sequences of other tetracycline resistance peptides were performed using Genetics Computer Group (GCG) software (Devereux, et al., *Nucl. Acids Res.,* 12:387–395 (1985)) on a MicroVAX computer system. The sequences of tetracycline resistance and elongation factor genes used in this study were obtained from GenBank and are listed with accession numbers in Table 3.

B. Results

1. Expression of the Bacteroides Tetracycline Resistance Gene in *E. coli*

The Tc^r gene from *B. thetaiotaomicron* DOT was localized to a 2.7 kbp SstI fragment in the constructs pNFD13-2 and pNFD13-6, which contained the insert in opposite orientations. Though these plasmids were

originally constructed to test for expression in Bacteroides, we examined them for expression in *E. coli* because it was possible that the lac promoter adjacent to the cloned SstI fragment would drive Tc^r expression in *E. coli*. Because *E. coli* carrying low copy number cosmid clones of the Tc^r gene did not grow on LB plates containing 5 or 10 μg/ml tetracycline, Shoemaker, et al. had reported that the *Bacteroides* Tc^r gene did not function in *E. coli* [Shoemaker, et al., *J. Bacteriol.*, 171:1294–1302 (1989)]. However, it was found that the 2.7 kbp SstI insert in the higher copy number pUC19-based vector, pFD160R, allowed *E. coli* to grow on LB agar plates containing 5 μg/ml tetracycline.

Following pregrowth in LB containing 100 μg/ml ampicillin, *E. coli* DH5α carrying the SstI clone had a tetracycline MIC value of 40 μg/ml for pNFD13-2 and a value of 25 μg/ml for pNFD13-6. However, when the inoculum culture was grown in LB containing sub-inhibitory tetracycline (1 μg/ml), differences in MIC between the clones diminished; the MIC values for pNFD13-2 and pNFD13-6 were 50 μg/ml and 40 μg/ml, respectively. The fact that the MIC values of both orientations were comparable indicated that the promoter being recognized was on the cloned fragment, and therefore was not the lac promoter. Moreover, addition of IPTG to the growth medium had no effect upon MIC levels. Interestingly, MIC values obtained on LB agar plates for *E. coli* bearing pNFD13-2 and pNFD13-6 were significantly lower than the values obtained in broth medium (plate MIC of 10 μg/ml for pNFD13-2).

2. Localization of the Bacteroides Tc^r Gene

Initially, two deletions in the 2.7 kbp SstI segment were created by digesting pNFD13-2 with EcoRV and religating to form pNFD13-2ΔRV and by digesting pNFD13-6 with EcoRI and religating to form pNFD13-6ΔRI. The MIC of DH5α bearing pNFD13-2ΔRV or pNFD13-6ΔRI was the same as that for DH5α without plasmid (2 μg/ml). Loss of resistance in both deletions indicated that the Tc^r gene spanned the internal 0.9 kbp EcoRI-EcoRV region of the SstI clone. Further localization of the gene was undertaken using exonuclease III to create progressive unidirectional deletions in the 2.7 kbp SstI insert from the pFD160 polylinker. (See FIG. 4) Deletion pNFD13-2Δ3, which extended from the right to within 100 bp of the EcoRI site, did not affect resistance in *E. coli*. Deletion pNFD13-2Δ4, which extended to within 50 bp of the EcoRI site, decreased the MIC without completely eliminating resistance. Deletions into or through the EcoRI site abolished Tc^r in *E. coli*. Deletion pNFD13-6Δ1, which extended 200 bp into the other end of the SstI fragment, also abolished Tc^r. Thus, it appeared that the genetic information essential for Tc^r expression in *E. coli* spanned a 2.1 kbp region in the SstI insert DNA.

A larger region was required for Tc^r expression in Bacteroides than in *E. coli*. Deletion construct pNFD13-2Δ3, which conferred full resistance on *E. coli*, did not confer resistance on Bacteroides. The largest of the exonuclease III deletions from the right which retained full Tc^r activity in Bacteroides was pNFD13-2Δ2. Thus, it appeared that an additional region of approximately 200 bp was required for expression in Bacteroides.

3. Size and Cellular Location of the Tc^r Gene Product

In maxicell experiments, two major proteins were associated with the cloned 2.7 kbp SstI fragment. These were estimated to have molecular weights of 76 and 25.5 kDa (data not shown). The two proteins were also seen when pNFD13-2 and its deletion derivatives were used as templates in an in vitro transcription-translation system. (See FIG. 5A) Appearance of the 76 kDa band coincided with Tc^r expression in *E. coli*. That is, the 76 kDa band was present in deletions that still conferred resistance on *E. coli* (pNFD13-2Δ1, pNFD13-2Δ3), was consistently fainter in the deletion which conferred reduced resistance (pNFD13-2Δ4), and was missing in the Tc^r deletions (pNFD13-2Δ5, pNFD13-2Δ6). By contrast, the 25.5 kDa band was produced from the Tc^s deletions pNFD13-2Δ5 and pNFD13-2Δ6. The Tc^r deletion in pNFD13-2ΔRV resulted in the loss of both of the major proteins associated with the SstI insert. Some additional proteins that were unique to the SstI clone were seen with the in vitro transcription. translation system, but these were also present in the Tc^s deletions pNFD13-2Δ5 and pNFD13-2Δ6. Moreover, these proteins were not seen in the maxicell extracts.

Cellular localization of the 76 kDa band by fractionation of maxicell extracts indicated that this protein partitioned predominately with the soluble fraction. (See FIG. 5B) However, a portion of the protein partitioned with the membrane fraction. The 25.5 kDa band clearly partitioned with the membrane fraction.

4 Relatedness to Other Bacteroides Tetracycline Resistances

Tetracycline resistance has been found to be widespread among strains of colonic Bacteroides. Previous hybridization studies of the Tc^r conjugal elements resident in different Tc^r colonic Bacteroides isolates have revealed extensive DNA hybridization [Shoemaker, et al., *J. Bacteriol.*, 171:1294–1302 (1989)]. To determine if the Tc^r genes in other clinical strains were similar to the Tc^r gene from *B. thetaiotaomicron* DOT, Southern hybridization was performed using the internal 0.9 kbp EcoRI-EcoRV segment of the Tc^r gene to probe chromosomal DNA preparations digested with EcoRV and EcoRI. The Tc^r strains analyzed were clinical isolates of *B. fragilis, B. thetaiotaomicron, B. uniformis, Bacteroides caccae,* and *Bacteroides distasonas* from the continental U.S., Hawaii and Japan. The 0.9 kbp probe hybridized with a 0.9 kbp band in all but one of the Tc^r isolates probed. The only exception to this was *B. distasonas* C30–45, in which the probe hybridized to a fragment much larger than 0.9 kbp. This could be due to the modification or loss of one of the two restriction sites in C30–45. In another *B. distasonas* isolate, 6308, the probe hybridized strongly to a 0.9 kbp band. *B. fragilis* V479 exhibited weak hybridization relative to the other strains, but the cross-hybridizing band was the same 0.9 kbp size as the probe. The probe did not hybridize to DNA from Tc^s type strain controls. These results indicated that the gene cloned in the 2.7 kbp SstI fragment is widespread among clinical isolates of colonic Bacteroides species. Given the stringency used in these experiments, it is estimated that the Tc^r genes found in other Tc^r strains of Bacteroides share at least 80% identity with the Tc^r gene from *B. thetaiotaomicron* DOT.

5. DNA Sequence

The DNA sequence of the 2.7 kbp SstI fragment was obtained. The sequence of the entire fragment is presented in Chart A below.

Only one open reading frame within the SstI clone was sufficiently large to encode a protein of the estimated 76 kDa. (See FIG. 4) All other open reading frames in the fragment were less than 400 bp. The start codon of the large open reading frame was 22 bp to the right of the EcoRI site in FIG. 1 The open reading frame spanned the 0.9 kbp EcoRI-EcoRV region, which was determined to be internal to the Tc^r gene. The location and extent of the open reading frame were also consistent with the exonuclease III deletion results. No additional open reading frames were found that might encode the 25.5 kDa protein seen in maxicells and in vitro transcription-translation. Presumably this protein was produced by a fusion between insert and vector DNA.

The TetQ open reading frame codes for a protein of 642 amino acids (deduced molecular weight, 72,100 Da). The amino acid sequence of the protein is given below in Chart B. The tetQ gene had 40.1 mol % G+C, compared to 42 mol % G+C of the chromosome of *B. thetaiotaomicron*, the species from which the Tc^r gene was cloned [Johnson, *J. Syst. Bacteriol.*, 28:245–256 (1979)].

6. Relatedness to Previously Sequenced Tc^r Proteins

The length of the deduced Bacteroides Tc^r protein was similar to the lengths of proteins encoded by tetM and tetO [Martin, et al., *Nucl. Acids Res.*, 14:7047–7058 (1986); Nesin, et al., *Antimicrob. Agents Chemother.*, in press; Sanchez-Pescador, et al., *Nucl. Acids Res.*, 16:1216–1217 (1988); LeBlanc, et al., *J. Bacteriol.*, 170:3618–3626 (1988); Manavathu, et al., *Gene.* 62:17–26 (1988)], which range from 638 amino acids to 640 amino acids. Comparisons of the Bacteroides Tc^r amino acid sequence to those of TetM and TetO revealed extensive regions of similarity. (See FIGS. 6A and 6B) However, the amino acid sequence of the Bacteroides Tc^r protein was less closely related to the amino acid sequences of TetM and TetO (40.1–40.3% identity) than these sequences are to each other (75.6–76.9% identity; Table 4). In these comparisons, clusters of identity extended over the length of the alignment, but were concentrated in the amino-terminal region. The amino acid sequence of the Bacteroides Tc^r protein had no significant similarity to those of sequenced Tc^r genes belonging either to the efflux or to the tetracycline detoxification classes of resistance. The results of these comparisons indicated the Bacteroides Tc^r gene was likely to be a member of that the ribosome protection class of Tc^r, but was clearly in a different hybridization class from TetM and TetO. Accordingly, we have designated this new class TetQ.

A hydrophobicity plot generated from the deduced amino acid sequence of TetQ was very similar to those generated for TetM and TetO. Since TetM and TetO are thought to be soluble proteins that function in the cytoplasm [Burdett, *J. Bacteriol.*, 165:564–569 (1986); Manavathu, et al., *Antimicrob. Agents Chemother.*, 34:71–77 (1990)], this suggests that TetQ is also a soluble protein. However, TetQ contained a relatively hydrophobic internal region (residues 205–247) that was not extant in TetM or TetO. This could explain why a portion of the Bacteroides Tc^r protein fractionated with the membrane in maxicell separations.

7. Upstream Region of tetQ

The DNA sequence of the upstream region of tetQ is shown in FIGS. 7A and 7B. An *E. coli*-like promoter sequence was found immediately upstream of the start of the open reading frame. The deletions in pNFD13.2Δ1 through pNFD13-2Δ3, which did not affect the tetracycline MIC in *E. coli*, left this promoter sequence intact. pNFD13-2Δ4, in which the −35 region of this promoter was deleted, reduced the MIC in *E. coli* pNFD13.2Δ5, in which both the −35 and the −10 region of this promoter were deleted, abolished resistance in *E. coli*. Thus, the *E. coli*-like −10 and −35 regions probably constitute the promoter that is driving transcription in *E. coli*.

Interestingly, this region was not sufficient for expression in Bacteroides, as evidenced by the observation that pNFD13-2Δ3 did not confer resistance on Bacteroides. The largest deletion that was still active in Bacteroides (pNFD13-2Δ2) contained the *E. coli* promoter plus about an additional 150 bp. The sequence of the smallest promoter region identified as functional in Bacteroides species is presented in Chart C below.

The upstream regions of tetM and tetO genes showed remarkable sequence similarity. This region contained the putative Gram-positive ribosome binding site [Martin, et al., *Nucl. Acids Res.*, 14:7047–7058 (1986)]. A comparison of the upstream region of tetM/O to that of tetQ disclosed no detectable similarity. (See FIGS. 6A and 6B) The tetQ upstream region also lacked a distinguishable ribosome binding site.

8. Relatedness to Tc^r of pRRI4

Plasmid pNFD13-2 labeled with P^{32} was used as a probe to hybridize to pRRI4 digested with EcoRI, PvuII, HincII-ECoRV and NciI. Plasmid pRRI4 in *P. ruminicola* 223/M2/7 was obtained from Dr. Harry J. Flint, Rowett Research Institute, Bucksburn, Aberdeen, U.K. It was extracted from *P. ruminicola* 223/M2/7 by standard techniques [Maniatis, et al., supra]. A cross-hybridizing region was identified. To ascertain if this cross-hybridizing region contained the Tc^r gene, a 5 kbp HincII-PvuII segment which covers this region was cloned into pFD160 and mobilized from *E. coli* into *B. uniformis*. The resulting transconjugants were Tc^r. Other hybridization experiments also indicated that the Tc^r gene on pRRI4 was at least 80% homologous to the Tc^r genes on pNFD13-2 and other Bacteroides Tc^{r}Em^r elements.

Recently, sequencing of the Tc^r gene on pRRI4 has been completed. Its sequence has been found to be 97% identical to that of the Tc^r gene on pNFD13-2. Accordingly, it is in the TetQ class.

C. Discussion

By size and amino acid sequence similarity, the Bacteroides TetQ appeared to be a ribosome protection type of tetracycline resistance. However, TetQ clearly did not belong in either class TetM or class TetO because the amino acid identity with those classes is only 40.3–40.9%.

All Tc^r Bacteroides strains that we screened had DNA which hybridized to an internal fragment of the cloned Tc^r gene under conditions of high stringency. Thus, TetQ is probably the predominant Tc^r among the colonic Bacteroides. In fact, recent evidence indicates that Tc^r determinants from colonic and oral Bacteroides have high similarity [Guiney and Bouic, *J. Bacteriol.*,

172:495–497 (1990)]. This suggests that TetQ may be ubiquitous in the genus Bacteroides. Also, at least one *P. ruminicola* Tc^r gene is of the TetQ class.

Previously sequenced ribosome protection Tc^r genes were remarkable for their sequence similarity (Table 4). For instance, the TetO found in *Campylobacter jejuni* shared 98.1% intraclass amino acid identity with the TetO found in *Streptococcus mutans*. The *C. jejuni* TetO shared 75.1–76.8% interclass amino acid identity with the TetM's found in Staphylococcus, Streptococcus, and Ureaplasma. Genes that are similar enough to cross-hybridize with tetM and tetO on Southern blots have been found in Clostridium, Eikenella, Fusobacterium, Gardnerella, Hemophilus, Kingella, Mycoplasma, Neisseria, and Veillonella [Salyers, et al., *Mol. Microbiol.*, 4:151–156 (1989)].

The amino-terminal regions of TetM and TetO have high amino acid similarity to the amino-terminal region of the elongation factors [Sanchez-Pescador, et al., *Nucl. Acids Res.*, 16, 1218 (1988); Manavathu et al., *Antimicrob. Agents Chemother.*, 34:71–77 (1990)]. This region is responsible for guanosine nucleotide binding by one elongation factor (EF-Tu) [Jacquet and Parmeggiani, *The EMBO J.*, 7:2861–2867 (1988); Jurnak, Science. 230:32–36 (1985)], and is conserved in GTP-binding proteins [Halliday, *J. Nucleotide Prot. Phosphoryl. Res.*, 9:435–448 (1984)]. Though TetQ is the most diverged ribosome protection Tc^r, it maintains high amino acid conservation in this GDP/GTP-binding domain. (See FIGS. 6A and 6B) This indicates that this functional domain may be involved directly in the ribosome protection resistance mechanism. Manavathu, et al., *Antimicrob. Agents Chemother.*, 34:71–77 (1990), suggested that TetO may have the potential to bind GTP, but no such binding has yet been demonstrated.

The upstream regions of tetM and tetO genes, which are virtually identical, contain a Gram-positive ribosome binding site. The mol % G+C of tetQ (40.1%) is similar to that of tetM and tetO, but is also similar to the mol % G+C of chromosomal DNA from colonic Bacteroides [39–46%; Johnson, *J. Syst. Bacteriol.*, 28:245–256 (1978)]. By contrast, the upstream region of tetO is completely different from that of tetM and tetO.

TABLE 2

Strains Used In Connection With Sequencing Of Tc^r

Strain or Plasmid	Relevant Phenotype
E. coli strains	
DH5α	RecA Δ(argF-lacA)U169 θ80dlacΔM15
LCD44	RecA MetE Tc^s derivative of RK5173.
Bacteroides strains	
B. thetaoitaomicron	
5482	
BT4001	Tc^s Em^s; Spontaneous Rif^r derivative of *B. thetaiotaomicron* 5482
BT4002	Tc^r
BT4004	Tc^r
BT4007	Tc^r Em^r
BT4008	Tc^r Em^r
2808	Tc^r
B. uniformis	
BU1001	Tc^s; Rif^r derivative of *B. uniformis* 0061
C7–17	Tc^r
3537	Tc^r
T1–1	Tc^r
B. distasonas	
4243	Tc^s
C30–45	Tc^r
6308	Tc^r
B. caccae	
3452A	Tc^s
8608	Tc^r
B. fragilis	
AK87	Tc^r

[a]Resistance phenotype expressed in *E. coli* is indicated in parenthesis.

TABLE 3

GenBank Access Codes For Sequences

Source organism	Gene product	GenBank Locus	GenBank Access.
Staphylococcus aureus	TetM	Statetm	M21136
Streptococcus faecalis	TetM	Str1545tr	X04388
Ureaplasma urealyticum	TetM	X06901	X06901
Campylobacter jejuni	TetO	Cajtrccra	M18896
Streptococcus mutans	TetO	Stateosm	M20925
Escherichia coli	EF-Tu	Ecotgtufb	J01717
	EF-G	Ecostra	X00415
Micrococcus luteus	EF-Tu	M17788	M17788
	EF-G	M17788	M17788
Spirulina platensis	EF-Tu	X15646	X15646
	EF-G	X15646	X15646
Thermus thermophilus	EF-Tu	Tthtuf1	X05977
	EF-G	X16278	X16278
Thermotoga maritima	EF-Tu	Tmoeftu	M27479
Euglena gracilis chloroplast	EF-Tu	Egrcpeftu	X00044
Methanococcus vannielii	EF-1	Mvatuf	X05698
	EF-2	Mvafus	X12384
Saccharomyces cerevisiae	EF-1α	Yscefla	X00779
Mucor racemonsus	EF-1α	Mratef1a	J02605
Dictyostelium discoideum	EF-2	Ddief2	M26017
Drosophila melanogaster	EF-2	X15805	X15805
Xenous laevis	EF-1α	Xelef1a1	M5697
Mesocricetus sp.	EF-2	Hamef2	M13708
Mus musculus	EF-1α	M22432	M22432
Rattus norvegicus	EF-2	Ratef2r	Y07504
Homo sapiens	EF-1α	Humef1a	X03558
	EF-2	Humef2ab	M30456

TABLE 4

Percent amino acid similarity and percent amino acid identity between deduced peptide sequences of ribosome protection tetracycline resistance genes

	percent similarity					
	1	2	3	4	5	6
1. *S. aureus* TetM	—	95.3	98.1	85.3	85.9	62.5
2. *S. pneumoniae* TetM	92.2	—	96.7	85.7	86.1	61.1
3. *U. urealyticum* TetM	96.6	95.0	—	85.7	86.3	62.5
4. *C. jejuni* TetO	75.1	76.8	76.0	—	98.4	60.5
5. *S. mutans* TetO	75.6	76.9	76.4	98.1	—	60.8
6. *B. thetaiotaomicron* TetQ	41.2	41.0	41.2	41.0	41.0	—
	percent identity					

Chart A

```
GAGCTCTAAA TTTAAATATA AACAACGAAT TATCTCCTTA ACGTACGTTT    50

TCGTTCCATT GGCCCTCAAA CCCCGTTATA TACATTCATG TCCATTTATG   100

TAAAAAATCC TGCTGACCTT GTTTATGTCT TGTCAGTCAC CATTTGCAAA   150

ACCATATTTG ACCCTCAAAG AGGCTGAATT TGATAAGCAA CTTGCTACAT   200

ACTCATAATA AGGAGCTAAA TAGAACACGA ATGGGAAATA CTCAAATGCC   250

AAACTAAAGA AGATATTGGC CAAAATAAAC GCTATACCGA GAGAGAAACT   300

TGATTTTTCA ACTTCCTAAA ACAGTGTTGT TCAAACATTT CTACTTATTT   350

GTACTTACCA GTTGAACCTA CGTTTCCCTA ATAAAATGTC TATGGTAAAA   400

AGTTAAAAAA TCCTCCTACT TTTGTTAGAT ATATTTTTTT GTGTAATTTT   450

GTAATCGTTA TGCGGCAGTA ATAATATACA TATTAATACG AGTTATTAAT   500

CCTGTAGTTC TCATATGCTA CGAGGAGGTA TTAAAAGGTG CGTTTCGACA   550

ATGCATCTAT TGTAGTATAT TATTGCTTAA TCCAA ATG AAT ATT ATA    597
                                      Met Asn Ile Ile

AAT TTA GGA ATT CTT GCT CAC ATT GAT GCA GGA AAA ACT TCC   639
Asn Leu Gly Ile Leu Ala His Ile Asp Ala Gly Lys Thr Ser
 5                  10                  15

GTA ACC GAG AAT CTG CTG TTT GCC AGT GGA GCA ACG GAA AAG   681
Val Thr Glu Asn Leu Leu Phe Ala Ser Gly Ala Thr Glu Lys
     20                  25                  30

TGC GGC TGT GTG GAT AAT GGT GAC ACC ATA ACG GAC TCT ATG   723
Cys Gly Cys Val Asp Asn Gly Asp Thr Ile Thr Asp Ser Met
     35                  40                  45

GAT ATA GAG AAA CGT AGA GGA ATT ACT GTT CGG GCT TCT ACG   765
Asp Ile Glu Lys Arg Arg Gly Ile Thr Val Arg Ala Ser Thr
     50                  55                  60

ACA TCT ATT ATC TGG AAT GGT GTG AAA TGC AAT ATC ATT GAC   807
Thr Ser Ile Ile Trp Asn Gly Val Lys Cys Asn Ile Ile Asp
     65                  70

ACT CCG GGA CAC ATG GAT TTT ATT GCG GAA GTG GAG CGG ACA   849
Thr Pro Gly His Met Asp Phe Ile Ala Glu Val Glu Arg Thr
 75                  80                  85

TTC AAA ATG CTT GAT GGA GCA GTC CTC ATC TTA TCC GCA AAG   891
Phe Lys Met Leu Asp Gly Ala Val Leu Ile Leu Ser Ala Lys
     90                  95                 100

GAA GGC ATA CAA GCG CAG ACA AAG TTG CTG TTC AAT ACT TTA   933
Glu Gly Ile Gln Ala Gln Thr Lys Leu Leu Phe Asn Thr Leu
    105                 110                 115

CAG AAG CTG CAA ATC CCG ACA ATT ATA TTT ATC AAT AAG ATT   975
Gln Lys Leu Gln Ile Pro Thr Ile Ile Phe Ile Asn Lys Ile
    120                 125                 130

GAC CGA GCC GGT GTG AAT TTG GAG CGT TTG TAT CTG GAT ATA   1017
Asp Arg Ala Gly Val Asn Leu Glu Arg Leu Tyr Leu Asp Ile
    135                 140

AAA GCA AAT CTG TCT CAA GAT GTC CTG TTT ATG CAA AAT GTT   1059
Lys Ala Asn Leu Ser Gln Asp Val Leu Phe Met Gln Asn Val
145                 150                 155

GTC GAT GGA TCG GTT TAT CCG GTT TGC TCC CAA ACA TAT ATA   1101
Val Asp Gly Ser Val Tyr Pro Val Cys Ser Gln Thr Tyr Ile
    160                 165                 170

AAG GAA GAA TAC AAA GAA TTT GTA TGC AAC CAT GAC GAC AAT   1143
Lys Glu Glu Tyr Lys Glu Phe Val Cys Asn His Asp Asp Asn
    175                 180                 185

ATA TTA GAA CGA TAT TTG GCG GAT AGC GAA ATT TCA CCG GCT   1185
Ile Leu Glu Arg Tyr Leu Ala Asp Ser Glu Ile Ser Pro Ala
    190                 195                 200
```

-continued

Chart A

GAT Asp	TAT Tyr	TGG Trp	AAT Asn	ACG Thr 205	ATA Ile	ATC Ile	GCT Ala	CTT Leu	GTG Val 210	GCA Ala	AAA Lys	GCC Ala	AAA Lys	1227
GTC Val 215	TAT Tyr	CCG Pro	GTG Val	CTA Leu	CAT His 220	GGA Gly	TCA Ser	GCA Ala	ATG Met	TTC Phe 225	AAT Asn	ATC Ile	GGT Gly	1269
ATC Ile	AAT Asn 230	GAG Glu	TTG Leu	TTG Leu	GAC Asp	GCC Ala 235	ATC Ile	ACT Thr	TCT Ser	TTT Phe	ATA Ile 240	CTT Leu	CCT Pro	1311
CCG Pro	GCA Ala	TCG Ser 245	GTC Val	TCA Ser	AAC Asn	AGA Arg	CTT Leu 250	TCA Ser	TCT Ser	TAT Tyr	CTT Leu	TCT Tyr 255	AAG Lys	1353
ATA Ile	GAG Glu	CAT His	GAC Asp 260	CCC Pro	AAA Lys	GGA Gly	CAT His	AAA Lys 265	AGA Arg	AGT Ser	TTT Phe	CTA Leu	AAA Lys 270	1395
ATA Ile	ATT Ile	GAC Asp	GGA Gly	AGT Ser 275	CTG Leu	AGA Arg	CTT Leu	CGA Arg	GAC Asp 280	GTT Val	GTA Val	AGA Arg	ATC Ile	1437
AAC Asn 285	GAT Asp	TCG Ser	GAA Glu	AAA Lys	TTC Phe 290	ATC Ile	AAG Lys	ATT Ile	AAA Lys	AAT Asn 295	CTA Leu	AAA Lys	ACT Thr	1479
ATC Ile	AAT Asn 300	CAG Gln	GGC Gly	AGA Arg	GAG Glu	ATA Ile 305	AAT Asn	GTT Val	GAT Asp	GAA Glu	GTG Val 310	GGC Gly	GCC Ala	1521
AAT Asn	GAT Asp	ATC Ile 315	GCG Ala	ATT Ile	GTA Val	GAG Glu	GAT Asp 320	ATG Met	GAT Asp	GAT Asp	TTT Phe	CGA Arg 325	ATC Ile	1563
GGA Gly	AAT Asn	TAT Tyr	TTA Leu 330	GGT Gly	GCT Ala	GAA Glu	CCT Pro	TGT Cys 335	TTG Leu	ATT Ile	CAA Gln	GGA Gly	TTA Leu 340	1605
TCG Ser	CAT His	CAG Gln	CAT His	CCC Pro 345	GCT Ala	CTC Leu	AAA Lys	TCC Ser	TCC Ser 350	GTC Val	CGG Arg	CCA Pro	GAC Asp	1647
AGG Arg 355	CCC Pro	GAA Glu	GAG Glu	AGA Arg	AGC Ser 360	AAG Lys	GTG Val	ATA Ile	TCC Ser	GCT Ala 365	CTG Leu	AAT Asn	ACA Thr	1689
TTG Leu	TGG Trp 370	ATT Ile	GAA Glu	GAC Asp	CCG Pro	TCT Ser 375	TTG Leu	TCC Ser	TTT Phe	TCC Ser	ATA Ile 380	AAC Asn	TCA Ser	1731
TAT Tyr	AGT Ser	GAT Asp 385	GAA Glu	TTG Leu	GAA Glu	ATC Ile	TCG Ser 390	TTA Leu	TAT Tyr	GGT Gly	TTA Leu	ACC Thr 395	CAA Gln	1773
AAG Lys	GAA Glu	ATC Ile	ATA Ile 400	CAG Gln	ACA Thr	TTG Leu	CTG Leu	GAA Glu 405	GAA Glu	CGA Arg	TTT Phe	TCC Ser	GTA Val 410	1815
AAG Lys	GTC Val	CAT His	TTT Phe	GAT Asp 415	GAG Glu	ATC Ile	AAG Lys	ACT Thr	ATA Ile 420	TAC Tyr	AAA Lys	GAA Glu	GGA Arg	1857
CCT Pro 425	GTA Val	AAA Lys	AAG Lys	GTC Val	AAT Asn 430	AAG Lys	ATT Ile	TAA Ile	CAG Gln	ATC Ile 435	GAA Glu	GTG Val	CCG Pro	1899
CCC Pro	AAC Asn 440	CCT Pro	TAT Tyr	TGG Trp	GCC Ala	ACA Thr 445	ATA Ile	GGG Gly	CTG Leu	ACT Thr	CTT Leu 450	GAT Glu	CCC Pro	1941
TTA Leu	CCG Pro	TTA Leu 455	GGG Gly	ACA Thr	GGG Gly	TTG Leu	CAA Gln 460	ATC Ile	GAA Glu	AGT Ser	GAC Asp	ATC Ile 465	TCC Ser	1983
TAT Tyr	GGT Gly	TAT Tyr	CTG Leu 470	AAC Asn	CAT His	TCT Ser	TTT Phe	CAA Gln 475	AAT Asn	GCC Ala	GTT Val	TTT Phe	GAA Glu 480	2025

-continued

Chart A

GGG Gly	ATT Ile	CGT Arg	ATG Met	TCT Ser 485	TGC Cys	CAA Gln	TCC Ser	GGG Gly	TTA Leu 490	CAT His	GGA Gly	TGG Trp	GAA Glu	2067
GTG Val 495	ACT Thr	GAT Asp	CTG Leu	AAA Lys	GTA Val 500	ACT Thr	TTT Phe	ACT Thr	CAA Gln	GCC Ala 505	GAG Glu	TAT Tyr	TAT Tyr	2109
AGC Ser	CCG Pro 510	GTA Val	AGT Ser	ACA Tyr	CCT Pro	GCT Ala 515	GAT Asp	TTC Phe	AGA Arg	CAG Gln	CTG Leu 520	ACC Thr	CCT Pro	2151
TAT Tyr	GTC Val	TTC Phe 525	AGG Arg	CTG Leu	GCC Ala	TTG Leu	CAA Gln 530	CAG Gln	TCA Ser	GGT Gly	GTG Val	GAC Asp 535	ATT Ile	2193
CTC Leu	GAA Glu	CCG Pro	ATG Met 540	CTC Leu	TAT Tyr	TTT Phe	GAG Glu	TTG Leu 545	CAG Gln	ATA Ile	CCC Pro	CAA Gln	GCG Ala 550	2235
GCA Ala	AGT Ser	TCC Ser	AAA Lys	GCT Ala 555	ATT Ile	ACA Thr	GAT Asp	TTG Leu	CAA Gln 560	AAA Lys	ATG Met	ATG Met	TCT Ser	2277
GAG Glu 565	ATT Ile	GAA Glu	GAC Asp	ATC Ile	AGT Ser 570	TGC Cys	AAT Asn	AAT Asn	GAG Glu	TGG Trp 575	TGT Cys	CAT His	ATT Ile	2319
AAA Lys	GGG Gly 580	AAA Lys	GTT Val	CCA Pro	TTA Leu	AAT Asn 585	ACA Thr	AGT Ser	AAA Lys	GAC Asp	TAT Tyr 590	GCA Ala	TAC Ser	2361
GAA Glu	GTA Val	AGT Ser 595	TCA Ser	TAC Tyr	ACT Thr	AAG Lys	GGC Gly 600	TTA Leu	GGC Gly	ATT Ile	TTT Phe	ATG Met 605	GTT Val	2403
AAG Lys	CCA Pro	TGC Cys	GGG Gly 610	TAT Tyr	CAA Gln	ATA Ile	ACA Thr	AAA Lys 615	GGC Gly	GGT Gly	TAT Tyr	TCT Ser	GAT Asp 620	2445
AAT Asn	ATC Ile	CGC Arg	ATG Met	AAC Asn 625	GAA Glu	AAA Lys	GAT Asp	AAA Lys	CTT Leu 630	TTA Leu	TTC Phe	ATG Met	TTC Phe	2487
CAA Gln 635	AAA Lys	TCA Ser	ATG Met	TCA Ser	TCA Ser 640	AAA Lys	TAATGGAGCG GTCAGGAAAT							2528

TTCTATAAGG CAATACAGTT GGGATATATA CTTATCTCCA TTCTTATCGG 2578

ATGTATGGCA TATAATAGCC TCTATGAATG GCAGGAGATA GAAGCATTAG 2628

AACTTGGCAA TAAAAAAATA GACGAGCTC 2657

(SEQ ID NO: 2)

Chart B

Met	Asn	Ile	Ile	Asn	Leu	Gly	Ile	Leu	Ala	His	Ile	(SEQ ID NO:3)			
				5				10						15	
Lys	Thr	Ser	Val	Thr	Glu	Asn	Leu	Leu	Phe	Ala	Ser	Gly	Ala	Thr	
				20				25						30	
Glu	Lys	Cys	Gly	Cys	Val	Asp	Asn	Gly	Asp	Thr	Ile	Thr	Asp	Ser	
				35				40						45	
Met	Asp	Ile	Glu	Lys	Arg	Arg	Gly	Ile	Thr	Val	Arg	Ala	Ser	Thr	
				50				55						60	
Thr	Ser	Ile	Ile	Trp	Asn	Gly	Val	Lys	Cys	Asn	Ile	Ile	Asp	Thr	
				65				70						75	
Pro	Gly	His	Met	Asp	Phe	Ile	Ala	Glu	Val	Glu	Arg	Thr	Phe	Lys	
				80				85						90	
Met	Leu	Asp	Gly	Ala	Val	Leu	Ile	Leu	Ser	Ala	Lys	Glu	Gly	Ile	
				95				100						105	

-continued

Chart B

Gln	Ala	Gln	Thr	Lys	Leu	Leu	Phe	Asn	Thr	Leu	Gln	Lys	Leu	Gln	
				110				115						120	
Ile	Pro	Thr	Ile	Ile	Phe	Ile	Asn	Lys	Ile	Asp	Arg	Ala	Gly	Val	
				125				130						135	
Asn	Leu	Glu	Arg	Leu	Tyr	Leu	Asp	Ile	Lys	Ala	Asn	Leu	Ser	Gln	
				140				145						150	
Asp	Val	Leu	Phe	Met	Gln	Asn	Val	Val	Asp	Gly	Ser	Val	Tyr	Pro	
				155				160						165	
Val	Cys	Ser	Gln	Thr	Tyr	Ile	Lys	Glu	Glu	Tyr	Lys	Glu	Phe	Val	
				170				175						180	
Cys	Asn	His	Asp	Asp	Asn	Ile	Leu	Glu	Arg	Tyr	Leu	Ala	Asp	Ser	
				185				190						195	
Glu	Ile	Ser	Pro	Ala	Asp	Tyr	Trp	Asn	Thr	Ile	Ile	Ala	Leu	Val	
				200				205						210	

-continued

Chart B

Ala Lys Ala Lys Val Tyr Pro Val Leu His Gly Ser Ala Met Phe
215 220 225

Asn Ile Gly Ile Asn Glu Leu Leu Asp Ala Ile Thr Ser Phe Ile
230 235 240

Leu Pro Pro Ala Ser Val Ser Asn Arg Leu Ser Ser Tyr Leu Tyr
245 250 255

Lys Ile Glu His Asp Pro Lys Gly His Lys Arg Ser Phe Leu Lys
260 265 270

Ile Ile Asp Gly Ser Leu Arg Leu Arg Asp Val Val Arg Ile Asn
275 280 285

Asp Ser Glu Lys Phe Ile Lys Ile Lys Asn Leu Lys Thr Ile Asn
290 295 300

Gln Gly Arg Glu Ile Asn Val Asp Glu Val Gly Ala Asn Asp Ile
305 310 315

Ala Ile Val Glu Asp Met Asp Asp Phe Arg Ile Gly Asn Tyr Leu
320 325 330

Gly Ala Glu Pro Cys Leu Ile Gln Gly Leu Ser His Gln His Pro
335 340 345

Ala Leu Lys Ser Ser Val Arg Pro Asp Arg Pro Glu Glu Arg Ser
350 355 360

Lys Val Ile Ser Ala Leu Asn Thr Leu Trp Ile Glu Asp Pro Ser
365 370 375

Leu Ser Phe Ser Ile Asn Ser Tyr Ser Asp Glu Leu Glu Ile Ser
380 385 390

Leu Tyr Gly Leu Thr Gln Lys Glu Ile Ile Gln Thr Leu Leu Glu
395 400 405

Glu Arg Phe Ser Val Lys Val His Phe Asp Glu Ile Lys Thr Ile
410 415 420

Tyr Lys Glu Arg Pro Val Lys Lys Val Asn Lys Ile Ile Gln Ile
425 430 435

-continued

Chart B

Glu Val Pro Pro Asn Pro Tyr Trp Ala Thr Ile Gly Leu Thr Leu
440 445 450

Glu Pro Leu Pro Leu Gly Thr Gly Leu Gln Ile Glu Ser Asp Ile
455 460 465

Ser Tyr Gly Tyr Leu Asn His Ser Phe Gln Asn Ala Val Phe Glu
470 475 480

Gly Ile Arg Met Ser Cys Gln Ser Gly Leu His Gly Trp Glu Val
485 490 495

Thr Asp Leu Lys Val Thr Phe Thr Gln Ala Glu Tyr Tyr Ser Pro
500 505 510

Val Ser Tyr Pro Ala Asp Phe Arg Gln Leu Thr Pro Tyr Val Phe
515 520 525

Arg Leu Ala Leu Gln Gln Ser Gly Val Asp Ile Leu Glu Pro Met
530 535 540

Leu Tyr Phe Glu Leu Gln Ile Pro Gln Ala Ala Ser Ser Lys Ala
545 550 555

Ile Thr Asp Leu Gln Lys Met Met Ser Glu Ile Glu Asp Ile Ser
560 565 570

Cys Asn Asn Glu Trp Cys His Ile Lys Gly Lys Val Pro Leu Asn
575 580 585

Thr Ser Lys Asp Tyr Ala Ser Glu Val Ser Ser Tyr Thr Lys Gly
590 595 600

Leu Gly Ile Phe Met Val Lys Pro Cys Gly Tyr Gln Ile Thr Lys
605 610 615

Gly Gly Tyr Ser Asp Asn Ile Arg Met Asn Glu Lys Asp Lys Leu
620 625 630

Leu Phe Met Phe Gln Lys Ser Met Ser Ser Lys
635 640

Chart C

```
AAAAATCCTC CTACTTTTGT TAGATATATT TTTTTGTGTA ATTTTGTAAT   50
CGTTATGCGG CAGTAATAAT ATACATATTA ATACGAGTTA TTAATCCTGT  100
AGTTCTCATA TGCTACGAGG AGGTATTAAA AGGTGCGTTT CGACAATGCA  150
TCTATTGTAG TATATTATTG CTTAATCCAA,                       180
```

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 5

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 180 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS:double stranded
(D) TOPOLOGY: circular

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
AAAAATCCTC CTACTTTTGT TAGATATATT TTTTTGTGTA ATTTTGTAAT   50
CGTTATGCGG CAGTAATAAT ATACATATTA ATACGAGTTA TTAATCCTGT  100
AGTTCTCATA TGCTACGAGG AGGTATTAAA AGGTGCGTTT CGACAATGCA  150
TCTATTGTAG TATATTATTG CTTAATCCAA                        180
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2657 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double stranded
(D) TOPOLOGY: circular

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
GAGCTCTAAA TTTAAATATA AACAACGAAT TATCTCCTTA ACGTACGTTT     50

TCGTTCCATT GGCCCTCAAA CCCCGTTATA TACATTCATG TCCATTTATG    100

TAAAAAATCC TGCTGACCTT GTTTATGTCT TGTCAGTCAC CATTTGCAAA    150

ACCATATTTG ACCCTCAAAG AGGCTGAATT TGATAAGCAA CTTGCTACAT    200

ACTCATAATA AGGAGCTAAA TAGAACACGA ATGGGAAATA CTCAAATGCC    250

AAACTAAAGA AGATATTGGC CAAAATAAAC GCTATACCGA GAGAGAAACT    300

TGATTTTTCA ACTTCCTAAA ACAGTGTTGT TCAAACATTT CTACTTATTT    350

GTACTTACCA GTTGAACCTA CGTTTCCCTA ATAAAATGTC TATGGTAAAA    400

AGTTAAAAAA TCCTCCTACT TTTGTTAGAT ATATTTTTTT GTGTAATTTT    450

GTAATCGTTA TGCGGCAGTA ATAATATACA TATTAATACG AGTTATTAAT    500

CCTGTAGTTC TCATATGCTA CGAGGAGGTA TTAAAAGGTG CGTTTCGACA    550

ATGCATCTAT TGTAGTATAT TATTGCTTAA TCCAA ATG AAT ATT ATA    597
                                      Met Asn Ile Ile

AAT TTA GGA ATT CTT GCT CAC ATT GAT GCA GGA AAA ACT TCC    639
Asn Leu Gly Ile Leu Ala His Ile Asp Ala Gly Lys Thr Ser
  5                  10                  15

GTA ACC GAG AAT CTG CTG TTT GCC AGT GGA GCA ACG GAA AAG    681
Val Thr Glu Asn Leu Leu Phe Ala Ser Gly Ala Thr Glu Lys
      20                  25                  30

TGC GGC TGT GTG GAT AAT GGT GAC ACC ATA ACG GAC TCT ATG    723
Cys Gly Cys Val Asp Asn Gly Asp Thr Ile Thr Asp Ser Met
          35                  40                  45

GAT ATA GAG AAA CGT AGA GGA ATT ACT GTT CGG GCT TCT ACG    765
Asp Ile Glu Lys Arg Arg Gly Ile Thr Val Arg Ala Ser Thr
              50                  55                  60

ACA TCT ATT ATC TGG AAT GGT GTG AAA TGC AAT ATC ATT GAC    807
Thr Ser Ile Ile Trp Asn Gly Val Lys Cys Asn Ile Ile Asp
          65                  70

ACT CCG GGA CAC ATG GAT TTT ATT GCG GAA GTG GAG CGG ACA    849
Thr Pro Gly His Met Asp Phe Ile Ala Glu Val Glu Arg Thr
 75                  80                  85

TTC AAA ATG CTT GAT GGA GCA GTC CTC ATC TTA TCC GCA AAG    891
Phe Lys Met Leu Asp Gly Ala Val Leu Ile Leu Ser Ala Lys
      90                  95                 100

GAA GGC ATA CAA GCG CAG ACA AAG TTG CTG TTC AAT ACT TTA    933
Glu Gly Ile Gln Ala Gln Thr Lys Leu Leu Phe Asn Thr Leu
         105                 110                 115

CAG AAG CTG CAA ATC CCG ACA ATT ATA TTT ATC AAT AAG ATT    975
Gln Lys Leu Gln Ile Pro Thr Ile Ile Phe Ile Asn Lys Ile
             120                 125                 130

GAC CGA GCC GGT GTG AAT TTG GAG CGT TTG TAT CTG GAT ATA   1017
Asp Arg Ala Gly Val Asn Leu Glu Arg Leu Tyr Leu Asp Ile
                 135                 140

AAA GCA AAT CTG TCT CAA GAT GTC CTG TTT ATG CAA AAT GTT   1059
Lys Ala Asn Leu Ser Gln Asp Val Leu Phe Met Gln Asn Val
145                 150                 155

GTC GAT GGA TCG GTT TAT CCG GTT TGC TCC CAA ACA TAT ATA   1101
Val Asp Gly Ser Val Tyr Pro Val Cys Ser Gln Thr Tyr Ile
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     160                 165                 170

AAG GAA GAA TAC AAA GAA TTT GTA TGC AAC CAT GAC GAC AAT 1143
Lys Glu Glu Tyr Lys Glu Phe Val Cys Asn His Asp Asp Asn
         175                 180                 185

ATA TTA GAA CGA TAT TTG GCG GAT AGC GAA ATT TCA CCG GCT 1185
Ile Leu Glu Arg Tyr Leu Ala Asp Ser Glu Ile Ser Pro Ala
             190                 195                 200

GAT TAT TGG AAT ACG ATA ATC GCT CTT GTG GCA AAA GCC AAA 1227
Asp Tyr Trp Asn Thr Ile Ile Ala Leu Val Ala Lys Ala Lys
                 205                 210

GTC TAT CCG GTG CTA CAT GGA TCA GCA ATG TTC AAT ATC GGT 1269
Val Tyr Pro Val Leu His Gly Ser Ala Met Phe Asn Ile Gly
215                 220                 225

ATC AAT GAG TTG TTG GAC GCC ATC ACT TCT TTT ATA CTT CCT 1311
Ile Asn Glu Leu Leu Asp Ala Ile Thr Ser Phe Ile Leu Pro
     230                 235                 240

CCG GCA TCG GTC TCA AAC AGA CTT TCA TCT TAT CTT TAT AAG 1353
Pro Ala Ser Val Ser Asn Arg Leu Ser Ser Tyr Leu Tyr Lys
         245                 250                 255

ATA GAG CAT GAC CCC AAA GGA CAT AAA AGA AGT TTT CTA AAA 1395
Ile Glu His Asp Pro Lys Gly His Lys Arg Ser Phe Leu Lys
             260                 265                 270

ATA ATT GAC GGA AGT CTG AGA CTT CGA GAC GTT GTA AGA ATC 1437
Ile Ile Asp Gly Ser Leu Arg Leu Arg Asp Val Val Arg Ile
                 275                 280

AAC GAT TCG GAA AAA TTC ATC AAG ATT AAA AAT CTA AAA ACT 1479
Asn Asp Ser Glu Lys Phe Ile Lys Ile Lys Asn Leu Lys Thr
285                 290                 295

ATC AAT CAG GGC AGA GAG ATA AAT GTT GAT GAA GTG GGC GCC 1521
Ile Asn Gln Gly Arg Glu Ile Asn Val Asp Glu Val Gly Ala
     300                 305                 310

AAT GAT ATC GCG ATT GTA GAG GAT ATG GAT GAT TTT CGA ATC 1563
Asn Asp Ile Ala Ile Val Glu Asp Met Asp Asp Phe Arg Ile
         315                 320                 325

GGA AAT TAT TTA GGT GCT GAA CCT TGT TTG ATT CAA GGA TTA 1605
Gly Asn Tyr Leu Gly Ala Glu Pro Cys Leu Ile Gln Gly Leu
             330                 335                 340

TCG CAT CAG CAT CCC GCT CTC AAA TCC TCC GTC CGG CCA GAC 1647
Ser His Gln His Pro Ala Leu Lys Ser Ser Val Arg Pro Asp
                 345                 350

AGG CCC GAA GAG AGA AGC AAG GTG ATA TCC GCT CTG AAT ACA 1689
Arg Pro Glu Glu Arg Ser Lys Val Ile Ser Ala Leu Asn Thr
355                 360                 365

TTG TGG ATT GAA GAC CCG TCT TTG TCC TTT TCC ATA AAC TCA 1731
Leu Trp Ile Glu Asp Pro Ser Leu Ser Phe Ser Ile Asn Ser
     370                 375                 380

TAT AGT GAT GAA TTG GAA ATC TCG TTA TAT GGT TTA ACC CAA 1773
Tyr Ser Asp Glu Leu Glu Ile Ser Leu Tyr Gly Leu Thr Gln
         385                 390                 395

AAG GAA ATC ATA CAG ACA TTG CTG GAA GAA CGA TTT TCC GTA 1815
Lys Glu Ile Ile Gln Thr Leu Leu Glu Glu Arg Phe Ser Val
             400                 405                 410

AAG GTC CAT TTT GAT GAG ATC AAG ACT ATA TAC AAA GAA GGA 1857
Lys Val His Phe Asp Glu Ile Lys Thr Ile Tyr Lys Glu Arg
                 415                 420

CCT GTA AAA AAG GTC AAT AAG ATT TAA CAG ATC GAA GTG CCG 1899
Pro Val Lys Lys Val Asn Lys Ile Ile Gln Ile Glu Val Pro
425                 430                 435

CCC AAC CCT TAT TGG GCC ACA ATA GGG CTG ACT CTT GAT CCC 1941
Pro Asn Pro Tyr Trp Ala Thr Ile Gly Leu Thr Leu Glu Pro
     440                 445                 450
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TTA CCG TTA GGG ACA GGG TTG CAA ATC GAA AGT GAC ATC TCC 1983
Leu Pro Leu Gly Thr Gly Leu Gln Ile Glu Ser Asp Ile Ser
        455                 460                 465

TAT GGT TAT CTG AAC CAT TCT TTT CAA AAT GCC GTT TTT GAA 2025
Tyr Gly Tyr Leu Asn His Ser Phe Gln Asn Ala Val Phe Glu
            470                 475                 480

GGG ATT CGT ATG TCT TGC CAA TCC GGG TTA CAT GGA TGG GAA 2067
Gly Ile Arg Met Ser Cys Gln Ser Gly Leu His Gly Trp Glu
                485                 490

GTG ACT GAT CTG AAA GTA ACT TTT ACT CAA GCC GAG TAT TAT 2109
Val Thr Asp Leu Lys Val Thr Phe Thr Gln Ala Glu Tyr Tyr
495                 500                 505

AGC CCG GTA AGT ACA CCT GCT GAT TTC AGA CAG CTG ACC CCT 2151
Ser Pro Val Ser Tyr Pro Ala Asp Phe Arg Gln Leu Thr Pro
    510                 515                 520

TAT GTC TTC AGG CTG GCC TTG CAA CAG TCA GGT GTG GAC ATT 2193
Tyr Val Phe Arg Leu Ala Leu Gln Gln Ser Gly Val Asp Ile
        525                 530                 535

CTC GAA CCG ATG CTC TAT TTT GAG TTG CAG ATA CCC CAA GCG 2235
Leu Glu Pro Met Leu Tyr Phe Glu Leu Gln Ile Pro Gln Ala
            540                 545                 550

GCA AGT TCC AAA GCT ATT ACA GAT TTG CAA AAA ATG ATG TCT 2277
Ala Ser Ser Lys Ala Ile Thr Asp Leu Gln Lys Met Met Ser
                555                 560

GAG ATT GAA GAC ATC AGT TGC AAT AAT GAG TGG TGT CAT ATT 2319
Glu Ile Glu Asp Ile Ser Cys Asn Asn Glu Trp Cys His Ile
565                 570                 575

AAA GGG AAA GTT CCA TTA AAT ACA AGT AAA GAC TAT GCA TCA 2361
Lys Gly Lys Val Pro Leu Asn Thr Ser Lys Asp Tyr Ala Ser
    580                 585                 590

GAA GTA AGT TCA TAC ACT AAG GGC TTA GGC ATT TTT ATG GTT 2403
Glu Val Ser Ser Tyr Thr Lys Gly Leu Gly Ile Phe Met Val
        595                 600                 605

AAG CCA TGC GGG TAT CAA ATA ACA AAA GGC GGT TAT TCT GAT 2445
Lys Pro Cys Gly Tyr Gln Ile Thr Lys Gly Gly Tyr Ser Asp
            610                 615                 620

AAT ATC CGC ATG AAC GAA AAA GAT AAA CTT TTA TTC ATG TTC 2487
Asn Ile Arg Met Asn Glu Lys Asp Lys Leu Leu Phe Met Phe
                625                 630

CAA AAA TCA ATG TCA TCA AAA TAATGGAGCG GTCAGGAAAT       2528
Gln Lys Ser Met Ser Ser Lys
635                 640

TTCTATAAGG CAATACAGTT GGGATATATA CTTATCTCCA TTCTTATCGG  2578

ATGTATGGCA TATAATAGCC TCTATGAATG GCAGGAGATA GAAGCATTAG  2628

AACTTGGCAA TAAAAAAATA GACGAGCTC                         2657
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 641 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Asn Ile Ile Asn Leu Gly Ile Leu Ala His Ile Asp Ala Gly
                5                  10                  15

Lys Thr Ser Val Thr Glu Asn Leu Leu Phe Ala Ser Gly Ala Thr
            20                  25                  30

Glu Lys Cys Gly Cys Val Asp Asn Gly Asp Thr Ile Thr Asp Ser
        35                  40                  45
```

-continued

Met Asp Ile Glu Lys Arg Arg Gly Ile Thr Val Arg Ala Ser Thr
50 55 60

Thr Ser Ile Ile Trp Asn Gly Val Lys Cys Asn Ile Ile Asp Thr
65 70 75

Pro Gly His Met Asp Phe Ile Ala Glu Val Glu Arg Thr Phe Lys
80 85 90

Met Leu Asp Gly Ala Val Leu Ile Leu Ser Ala Lys Glu Gly Ile
95 100 105

Gln Ala Gln Thr Lys Leu Leu Phe Asn Thr Leu Gln Lys Leu Gln
110 115 120

Ile Pro Thr Ile Ile Phe Ile Asn Lys Ile Asp Arg Ala Gly Val
125 130 135

Asn Leu Glu Arg Leu Tyr Leu Asp Ile Lys Ala Asn Leu Ser Gln
140 145 150

Asp Val Leu Phe Met Gln Asn Val Val Asp Gly Ser Val Tyr Pro
155 160 165

Val Cys Ser Gln Thr Tyr Ile Lys Glu Glu Tyr Lys Glu Phe Val
170 175 180

Cys Asn His Asp Asp Asn Ile Leu Glu Arg Tyr Leu Ala Asp Ser
185 190 195

Glu Ile Ser Pro Ala Asp Tyr Trp Asn Thr Ile Ile Ala Leu Val
200 205 210

Ala Lys Ala Lys Val Tyr Pro Val Leu His Gly Ser Ala Met Phe
215 220 225

Asn Ile Gly Ile Asn Glu Leu Leu Asp Ala Ile Thr Ser Phe Ile
230 235 240

Leu Pro Pro Ala Ser Val Ser Asn Arg Leu Ser Ser Tyr Leu Tyr
245 250 255

Lys Ile Glu His Asp Pro Lys Gly His Lys Arg Ser Phe Leu Lys
260 265 270

Ile Ile Asp Gly Ser Leu Arg Leu Arg Asp Val Val Arg Ile Asn
275 280 285

Asp Ser Glu Lys Phe Ile Lys Ile Lys Asn Leu Lys Thr Ile Asn
290 295 300

Gln Gly Arg Glu Ile Asn Val Asp Glu Val Gly Ala Asn Asp Ile
305 310 315

Ala Ile Val Glu Asp Met Asp Asp Phe Arg Ile Gly Asn Tyr Leu
320 325 330

Gly Ala Glu Pro Cys Leu Ile Gln Gly Leu Ser His Gln His Pro
335 340 345

Ala Leu Lys Ser Ser Val Arg Pro Asp Arg Pro Glu Glu Arg Ser
350 355 360

Lys Val Ile Ser Ala Leu Asn Thr Leu Trp Ile Glu Asp Pro Ser
365 370 375

Leu Ser Phe Ser Ile Asn Ser Tyr Ser Asp Glu Leu Glu Ile Ser
380 385 390

Leu Tyr Gly Leu Thr Gln Lys Glu Ile Ile Gln Thr Leu Leu Glu
395 400 405

Glu Arg Phe Ser Val Lys Val His Phe Asp Glu Ile Lys Thr Ile
410 415 420

Tyr Lys Glu Arg Pro Val Lys Lys Val Asn Lys Ile Ile Gln Ile
425 430 435

Glu Val Pro Pro Asn Pro Tyr Trp Ala Thr Ile Gly Leu Thr Leu
440 445 450

Glu Pro Leu Pro Leu Gly Thr Gly Leu Gln Ile Glu Ser Asp Ile

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                455                 460                 465

Ser Tyr Gly Tyr Leu Asn His Ser Phe Gln Asn Ala Val Phe Glu
                470                 475                 480

Gly Ile Arg Met Ser Cys Gln Ser Gly Leu His Gly Trp Glu Val
                485                 490                 495

Thr Asp Leu Lys Val Thr Phe Thr Gln Ala Glu Tyr Tyr Ser Pro
                500                 505                 510

Val Ser Tyr Pro Ala Asp Phe Arg Gln Leu Thr Pro Tyr Val Phe
                515                 520                 525

Arg Leu Ala Leu Gln Gln Ser Gly Val Asp Ile Leu Glu Pro Met
                530                 535                 540

Leu Tyr Phe Glu Leu Gln Ile Pro Gln Ala Ala Ser Ser Lys Ala
                545                 550                 555

Ile Thr Asp Leu Gln Lys Met Met Ser Glu Ile Glu Asp Ile Ser
                560                 565                 570

Cys Asn Asn Glu Trp Cys His Ile Lys Gly Lys Val Pro Leu Asn
                575                 580                 585

Thr Ser Lys Asp Tyr Ala Ser Glu Val Ser Ser Tyr Thr Lys Gly
                590                 595                 600

Leu Gly Ile Phe Met Val Lys Pro Cys Gly Tyr Gln Ile Thr Lys
                605                 610                 615

Gly Gly Tyr Ser Asp Asn Ile Arg Met Asn Glu Lys Asp Lys Leu
                620                 625                 630

Leu Phe Met Phe Gln Lys Ser Met Ser Ser Lys
                635                 640
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2106 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double stranded
(D) TOPOLOGY: circular

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
AAAAATCCTC CTACTTTTGT TAGATATATT TTTTTGTGTA ATTTTGTAAT          50

CGTTATGCGG CAGTAATAAT ATACATATTA ATACGAGTTA GGAATCCTGT         100

AGTTCTCATA TGCTACGAGG AGGTATTAAA AGGTGCGTTT CGACAATGCA         150

TCTATTGTAG TATATTATTG CTTAATCCAA ATG AAT ATT ATA               192
                                  Met Asn Ile Ile

AAT TTA GGA ATT CTT GCT CAC ATT GAT GCA GGA AAA ACT TCC        234
Asn Leu Gly Ile Leu Ala His Ile Asp Ala Gly Lys Thr Ser
  5                 10                  15

GTA ACC GAG AAT CTG CTG TTT GCC AGT GGA GCA ACG GAA AAG        276
Val Thr Glu Asn Leu Leu Phe Ala Ser Gly Ala Thr Glu Lys
      20                  25                  30

TGC GGC TGT GTG GAT AAT GGT GAC ACC ATA ACG GAC TCT ATG        318
Cys Gly Cys Val Asp Asn Gly Asp Thr Ile Thr Asp Ser Met
          35                  40                  45

GAT ATA GAG AAA CGT AGA GGA ATT ACT GTT CGG GCT TCT ACG        360
Asp Ile Glu Lys Arg Arg Gly Ile Thr Val Arg Ala Ser Thr
              50                  55                  60

ACA TCT ATT ATC TGG AAT GGT GTG AAA TGC AAT ATC ATT GAC        402
Thr Ser Ile Ile Trp Asn Gly Val Lys Cys Asn Ile Ile Asp
                  65                  70

ACT CCG GGA CAC ATG GAT TTT ATT GCG GAA GTG GAG CGG ACA        444
Thr Pro Gly His Met Asp Phe Ile Ala Glu Val Glu Arg Thr
  75                  80                  85
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TTC AAA ATG CTT GAT GGA GCA GTC CTC ATC TTA TCC GCA AAG      486
Phe Lys Met Leu Asp Gly Ala Val Leu Ile Leu Ser Ala Lys
 90                  95                 100

GAA GGC ATA CAA GCG CAG ACA AAG TTG CTG TTC AAT ACT TTA      528
Glu Gly Ile Gln Ala Gln Thr Lys Leu Leu Phe Asn Thr Leu
105                 110                 115

CAG AAG CTG CAA ATC CCG ACA ATT ATA TTT ATC AAT AAG ATT      570
Gln Lys Leu Gln Ile Pro Thr Ile Ile Phe Ile Asn Lys Ile
120                 125                 130

GAC CGA GCC GGT GTG AAT TTG GAG CGT TTG TAT CTG GAT ATA      612
Asp Arg Ala Gly Val Asn Leu Glu Arg Leu Tyr Leu Asp Ile
135                 140

AAA GCA AAT CTG TCT CAA GAT GTC CTG TTT ATG CAA AAT GTT      654
Lys Ala Asn Leu Ser Gln Asp Val Leu Phe Met Gln Asn Val
145                 150                 155

GTC GAT GGA TCG GTT TAT CCG GTT TGC TCC CAA ACA TAT ATA      696
Val Asp Gly Ser Val Tyr Pro Val Cys Ser Gln Thr Tyr Ile
160                 165                 170

AAG GAA GAA TAC AAA GAA TTT GTA TGC AAC CAT GAC GAC AAT      738
Lys Glu Glu Tyr Lys Glu Phe Val Cys Asn His Asp Asp Asn
175                 180                 185

ATA TTA GAA CGA TAT TTG GCG GAT AGC GAA ATT TCA CCG GCT      780
Ile Leu Glu Arg Tyr Leu Ala Asp Ser Glu Ile Ser Pro Ala
190                 195                 200

GAT TAT TGG AAT ACG ATA ATC GCT CTT GTG GCA AAA GCC AAA      822
Asp Tyr Trp Asn Thr Ile Ile Ala Leu Val Ala Lys Ala Lys
205                 210

GTC TAT CCG GTG CTA CAT GGA TCA GCA ATG TTC AAT ATC GGT      864
Val Tyr Pro Val Leu His Gly Ser Ala Met Phe Asn Ile Gly
215                 220                 225

ATC AAT GAG TTG TTG GAC GCC ATC ACT TCT TTT ATA CTT CCT      906
Ile Asn Glu Leu Leu Asp Ala Ile Thr Ser Phe Ile Leu Pro
230                 235                 240

CCG GCA TCG GTC TCA AAC AGA CTT TCA TCT TAT CTT TAT AAG      948
Pro Ala Ser Val Ser Asn Arg Leu Ser Ser Tyr Leu Tyr Lys
245                 250                 255

ATA GAG CAT GAC CCC AAA GGA CAT AAA AGA AGT TTT CTA AAA      990
Ile Glu His Asp Pro Lys Gly His Lys Arg Ser Phe Leu Lys
260                 265                 270

ATA ATT GAC GGA AGT CTG AGA CTT CGA GAC GTT GTA AGA ATC     1032
Ile Ile Asp Gly Ser Leu Arg Leu Arg Asp Val Val Arg Ile
275                 280

AAC GAT TCG GAA AAA TTC ATC AAG ATT AAA AAT CTA AAA ACT     1074
Asn Asp Ser Glu Lys Phe Ile Lys Ile Lys Asn Leu Lys Thr
285                 290                 295

ATC AAT CAG GGC AGA GAG ATA AAT GTT GAT GAA GTG GGC GCC     1116
Ile Asn Gln Gly Arg Glu Ile Asn Val Asp Glu Val Gly Ala
300                 305                 310

AAT GAT ATC GCG ATT GTA GAG GAT ATG GAT GAT TTT CGA ATC     1158
Asn Asp Ile Ala Ile Val Glu Asp Met Asp Asp Phe Arg Ile
315                 320                 325

GGA AAT TAT TTA GGT GCT GAA CCT TGT TTG ATT CAA GGA TTA     1200
Gly Asn Tyr Leu Gly Ala Glu Pro Cys Leu Ile Gln Gly Leu
330                 335                 340

TCG CAT CAG CAT CCC GCT CTC AAA TCC TCC GTC CGG CCA GAC     1242
Ser His Gln His Pro Ala Leu Lys Ser Ser Val Arg Pro Asp
345                 350

AGG CCC GAA GAG AGA AGC AAG GTG ATA TCC GCT CTG AAT ACA     1284
Arg Pro Glu Glu Arg Ser Lys Val Ile Ser Ala Leu Asn Thr
355                 360                 365

TTG TGG ATT GAA GAC CCG TCT TTG TCC TTT TCC ATA AAC TCA     1326
Leu Trp Ile Glu Asp Pro Ser Leu Ser Phe Ser Ile Asn Ser
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     370                 375                 380
TAT AGT GAT GAA TTG GAA ATC TCG TTA TAT GGT TTA ACC CAA   1368
Tyr Ser Asp Glu Leu Glu Ile Ser Leu Tyr Gly Leu Thr Gln
        385                 390                 395

AAG GAA ATC ATA CAG ACA TTG CTG GAA GAA CGA TTT TCC GTA   1410
Lys Glu Ile Ile Gln Thr Leu Leu Glu Glu Arg Phe Ser Val
            400                 405                 410

AAG GTC CAT TTT GAT GAG ATC AAG ACT ATA TAC AAA GAA GGA   1452
Lys Val His Phe Asp Glu Ile Lys Thr Ile Tyr Lys Glu Arg
                415                 420

CCT GTA AAA AAG GTC AAT AAG ATT TAA CAG ATC GAA GTG CCG   1494
Pro Val Lys Lys Val Asn Lys Ile Ile Gln Ile Glu Val Pro
425                 430                 435

CCC AAC CCT TAT TGG GCC ACA ATA GGG CTG ACT CTT GAT CCC   1536
Pro Asn Pro Tyr Trp Ala Thr Ile Gly Leu Thr Leu Glu Pro
    440                 445                 450

TTA CCG TTA GGG ACA GGG TTG CAA ATC GAA AGT GAC ATC TCC   1578
Leu Pro Leu Gly Thr Gly Leu Gln Ile Glu Ser Asp Ile Ser
        455                 460                 465

TAT GGT TAT CTG AAC CAT TCT TTT CAA AAT GCC GTT TTT GAA   1620
Tyr Gly Tyr Leu Asn His Ser Phe Gln Asn Ala Val Phe Glu
            470                 475                 480

GGG ATT CGT ATG TCT TGC CAA TCC GGG TTA CAT GGA TGG GAA   1662
Gly Ile Arg Met Ser Cys Gln Ser Gly Leu His Gly Trp Glu
                485                 490

GTG ACT GAT CTG AAA GTA ACT TTT ACT CAA GCC GAG TAT TAT   1704
Val Thr Asp Leu Lys Val Thr Phe Thr Gln Ala Glu Tyr Tyr
495                 500                 505

AGC CCG GTA AGT ACA CCT GCT GAT TTC AGA CAG CTG ACC CCT   1746
Ser Pro Val Ser Tyr Pro Ala Asp Phe Arg Gln Leu Thr Pro
    510                 515                 520

TAT GTC TTC AGG CTG GCC TTG CAA CAG TCA GGT GTG GAC ATT   1788
Tyr Val Phe Arg Leu Ala Leu Gln Gln Ser Gly Val Asp Ile
        525                 530                 535

CTC GAA CCG ATG CTC TAT TTT GAG TTG CAG ATA CCC CAA GCG   1830
Leu Glu Pro Met Leu Tyr Phe Glu Leu Gln Ile Pro Gln Ala
            540                 545                 550

GCA AGT TCC AAA GCT ATT ACA GAT TTG CAA AAA ATG ATG TCT   1872
Ala Ser Ser Lys Ala Ile Thr Asp Leu Gln Lys Met Met Ser
                555                 560

GAG ATT GAA GAC ATC AGT TGC AAT AAT GAG TGG TGT CAT ATT   1914
Glu Ile Glu Asp Ile Ser Cys Asn Asn Glu Trp Cys His Ile
565                 570                 575

AAA GGG AAA GTT CCA TTA AAT ACA AGT AAA GAC TAT GCA TCA   1956
Lys Gly Lys Val Pro Leu Asn Thr Ser Lys Asp Tyr Ala Ser
    580                 585                 590

GAA GTA AGT TCA TAC ACT AAG GGC TTA GGC ATT TTT ATG GTT   1998
Glu Val Ser Ser Tyr Thr Lys Gly Leu Gly Ile Phe Met Val
        595                 600                 605

AAG CCA TGC GGG TAT CAA ATA ACA AAA GGC GGT TAT TCT GAT   2040
Lys Pro Cys Gly Tyr Gln Ile Thr Lys Gly Gly Tyr Ser Asp
            610                 615                 620

AAT ATC CGC ATG AAC GAA AAA GAT AAA CTT TTA TTC ATG TTC   2082
Asn Ile Arg Met Asn Glu Lys Asp Lys Leu Leu Phe Met Phe
                625                 630

CAA AAA TCA ATG TCA TCA AAA TAA                           2106
Gln Lys Ser Met Ser Ser Lys
635                 640
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 1926 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double stranded
(D) TOPOLOGY: circular

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ATG AAT ATT ATA AAT TTA GGA ATT CTT GCT CAC ATT GAT GCA    42
Met Asn Ile Ile Asn Leu Gly Ile Leu Ala His Ile Asp Ala
                 5                  10

GGA AAA ACT TCC GTA ACC GAG AAT CTG CTG TTT GCC AGT GGA    84
Gly Lys Thr Ser Val Thr Glu Asn Leu Leu Phe Ala Ser Gly
 15                  20                  25

GCA ACG GAA AAG TGC GGC TGT GTG GAT AAT GGT GAC ACC ATA   126
Ala Thr Glu Lys Cys Gly Cys Val Asp Asn Gly Asp Thr Ile
      30                  35                  40

ACG GAC TCT ATG GAT ATA GAG AAA CGT AGA GGA ATT ACT GTT   168
Thr Asp Ser Met Asp Ile Glu Lys Arg Arg Gly Ile Thr Val
          45                  50                  55

CGG GCT TCT ACG ACA TCT ATT ATC TGG AAT GGT GTG AAA TGC   210
Arg Ala Ser Thr Thr Ser Ile Ile Trp Asn Gly Val Lys Cys
              60                  65                  70

AAT ATC ATT GAC ACT CCG GGA CAC ATG GAT TTT ATT GCG GAA   252
Asn Ile Ile Asp Thr Pro Gly His Met Asp Phe Ile Ala Glu
                  75                  80

GTG GAG CGG ACA TTC AAA ATG CTT GAT GGA GCA GTC CTC ATC   294
Val Glu Arg Thr Phe Lys Met Leu Asp Gly Ala Val Leu Ile
 85                  90                  95

TTA TCC GCA AAG GAA GGC ATA CAA GCG CAG ACA AAG TTG CTG   336
Leu Ser Ala Lys Glu Gly Ile Gln Ala Gln Thr Lys Leu Leu
     100                 105                 110

TTC AAT ACT TTA CAG AAG CTG CAA ATC CCG ACA ATT ATA TTT   378
Phe Asn Thr Leu Gln Lys Leu Gln Ile Pro Thr Ile Ile Phe
         115                 120                 125

ATC AAT AAG ATT GAC CGA GCC GGT GTG AAT TTG GAG CGT TTG   420
Ile Asn Lys Ile Asp Arg Ala Gly Val Asn Leu Glu Arg Leu
             130                 135                 140

TAT CTG GAT ATA AAA GCA AAT CTG TCT CAA GAT GTC CTG TTT   462
Tyr Leu Asp Ile Lys Ala Asn Leu Ser Gln Asp Val Leu Phe
                 145                 150

ATG CAA AAT GTT GTC GAT GGA TCG GTT TAT CCG GTT TGC TCC   504
Met Gln Asn Val Val Asp Gly Ser Val Tyr Pro Val Cys Ser
155                 160                 165

CAA ACA TAT ATA AAG GAA GAA TAC AAA GAA TTT GTA TGC AAC   546
Gln Thr Tyr Ile Lys Glu Glu Tyr Lys Glu Phe Val Cys Asn
     170                 175                 180

CAT GAC GAC AAT ATA TTA GAA CGA TAT TTG GCG GAT AGC GAA   588
His Asp Asp Asn Ile Leu Glu Arg Tyr Leu Ala Asp Ser Glu
         185                 190                 195

ATT TCA CCG GCT GAT TAT TGG AAT ACG ATA ATC GCT CTT GTG   630
Ile Ser Pro Ala Asp Tyr Trp Asn Thr Ile Ile Ala Leu Val
             200                 205                 210

GCA AAA GCC AAA GTC TAT CCG GTG CTA CAT GGA TCA GCA ATG   672
Ala Lys Ala Lys Val Tyr Pro Val Leu His Gly Ser Ala Met
                 215                 220

TTC AAT ATC GGT ATC AAT GAG TTG TTG GAC GCC ATC ACT TCT   714
Phe Asn Ile Gly Ile Asn Glu Leu Leu Asp Ala Ile Thr Ser
225                 230                 235

TTT ATA CTT CCT CCG GCA TCG GTC TCA AAC AGA CTT TCA TCT   756
Phe Ile Leu Pro Pro Ala Ser Val Ser Asn Arg Leu Ser Ser
     240                 245                 250

TAT CTT TAT AAG ATA GAG CAT GAC CCC AAA GGA CAT AAA AGA   798
Tyr Leu Tyr Lys Ile Glu His Asp Pro Lys Gly His Lys Arg
         255                 260                 265
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-continued

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AGT TTT CTA AAA ATA ATT GAC GGA AGT CTG AGA CTT CGA GAC  840
Ser Phe Leu Lys Ile Ile Asp Gly Ser Leu Arg Leu Arg Asp
                270                 275                 280

GTT GTA AGA ATC AAC GAT TCG GAA AAA TTC ATC AAG ATT AAA  882
Val Val Arg Ile Asn Asp Ser Glu Lys Phe Ile Lys Ile Lys
            285                 290

AAT CTA AAA ACT ATC AAT CAG GGC AGA GAG ATA AAT GTT GAT  924
Asn Leu Lys Thr Ile Asn Gln Gly Arg Glu Ile Asn Val Asp
295                 300                 305

GAA GTG GGC GCC AAT GAT ATC GCG ATT GTA GAG GAT ATG GAT  966
Glu Val Gly Ala Asn Asp Ile Ala Ile Val Glu Asp Met Asp
        310                 315                 320

GAT TTT CGA ATC GGA AAT TAT TTA GGT GCT GAA CCT TGT TTG 1008
Asp Phe Arg Ile Gly Asn Tyr Leu Gly Ala Glu Pro Cys Leu
            325                 330                 335

ATT CAA GGA TTA TCG CAT CAG CAT CCC GCT CTC AAA TCC TCC 1050
Ile Gln Gly Leu Ser His Gln His Pro Ala Leu Lys Ser Ser
                340                 345                 350

GTC CGG CCA GAC AGG CCC GAA GAG AGA AGC AAG GTG ATA TCC 1092
Val Arg Pro Asp Arg Pro Glu Glu Arg Ser Lys Val Ile Ser
            355                 360

GCT CTG AAT ACA TTG TGG ATT GAA GAC CCG TCT TTG TCC TTT 1134
Ala Leu Asn Thr Leu Trp Ile Glu Asp Pro Ser Leu Ser Phe
365                 370                 375

TCC ATA AAC TCA TAT AGT GAT GAA TTG GAA ATC TCG TTA TAT 1176
Ser Ile Asn Ser Tyr Ser Asp Glu Leu Glu Ile Ser Leu Tyr
        380                 385                 390

GGT TTA ACC CAA AAG GAA ATC ATA CAG ACA TTG CTG GAA GAA 1218
Gly Leu Thr Gln Lys Glu Ile Ile Gln Thr Leu Leu Glu Glu
            395                 400                 405

CGA TTT TCC GTA AAG GTC CAT TTT GAT GAG ATC AAG ACT ATA 1260
Arg Phe Ser Val Lys Val His Phe Asp Glu Ile Lys Thr Ile
                410                 415                 420

TAC AAA GAA GGA CCT GTA AAA AAG GTC AAT AAG ATT TAA CAG 1302
Tyr Lys Glu Arg Pro Val Lys Lys Val Asn Lys Ile Ile Gln
            425                 430

ATC GAA GTG CCG CCC AAC CCT TAT TGG GCC ACA ATA GGG CTG 1344
Ile Glu Val Pro Pro Asn Pro Tyr Trp Ala Thr Ile Gly Leu
435                 440                 445

ACT CTT GAT CCC TTA CCG TTA GGG ACA GGG TTG CAA ATC GAA 1386
Thr Leu Glu Pro Leu Pro Leu Gly Thr Gly Leu Gln Ile Glu
        450                 455                 460

AGT GAC ATC TCC TAT GGT TAT CTG AAC CAT TCT TTT CAA AAT 1428
Ser Asp Ile Ser Tyr Gly Tyr Leu Asn His Ser Phe Gln Asn
            465                 470                 475

GCC GTT TTT GAA GGG ATT CGT ATG TCT TGC CAA TCC GGG TTA 1470
Ala Val Phe Glu Gly Ile Arg Met Ser Cys Gln Ser Gly Leu
                480                 485                 490

CAT GGA TGG GAA GTG ACT GAT CTG AAA GTA ACT TTT ACT CAA 1512
His Gly Trp Glu Val Thr Asp Leu Lys Val Thr Phe Thr Gln
            495                 500

GCC GAG TAT TAT AGC CCG GTA AGT ACA CCT GCT GAT TTC AGA 1554
Ala Glu Tyr Tyr Ser Pro Val Ser Tyr Pro Ala Asp Phe Arg
505                 510                 515

CAG CTG ACC CCT TAT GTC TTC AGG CTG GCC TTG CAA CAG TCA 1596
Gln Leu Thr Pro Tyr Val Phe Arg Leu Ala Leu Gln Gln Ser
        520                 525                 530

GGT GTG GAC ATT CTC GAA CCG ATG CTC TAT TTT GAG TTG CAG 1638
Gly Val Asp Ile Leu Glu Pro Met Leu Tyr Phe Glu Leu Gln
            535                 540                 545

ATA CCC CAA GCG GCA AGT TCC AAA GCT ATT ACA GAT TTG CAA 1680
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-continued

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Ile Pro Gln Ala Ala Ser Ser Lys Ala Ile Thr Asp Leu Gln
          550                 555                 560

AAA ATG ATG TCT GAG ATT GAA GAC ATC AGT TGC AAT AAT GAG 1722
Lys Met Met Ser Glu Ile Glu Asp Ile Ser Cys Asn Asn Glu
              565                 570

TGG TGT CAT ATT AAA GGG AAA GTT CCA TTA AAT ACA AGT AAA 1764
Trp Cys His Ile Lys Gly Lys Val Pro Leu Asn Thr Ser Lys
575               580                 585

GAC TAT GCA TCA GAA GTA AGT TCA TAC ACT AAG GGC TTA GGC 1806
Asp Tyr Ala Ser Glu Val Ser Ser Tyr Thr Lys Gly Leu Gly
     590                 595                 600

ATT TTT ATG GTT AAG CCA TGC GGG TAT CAA ATA ACA AAA GGC 1848
Ile Phe Met Val Lys Pro Cys Gly Tyr Gln Ile Thr Lys Gly
         605                 610                 615

GGT TAT TCT GAT AAT ATC CGC ATG AAC GAA AAA GAT AAA CTT 1890
Gly Tyr Ser Asp Asn Ile Arg Met Asn Glu Lys Asp Lys Leu
              620                 625                 630

TTA TTC ATG TTC CAA AAA TCA ATG TCA TCA AAA TAA         1926
Leu Phe Met Phe Gln Lys Ser Met Ser Ser Lys
              635                 640
```

We claim:

1. A method of introducing heterologous DNA into a *Prevotella ruminicola,* comprising:

(a) transforming an *Escherichia coli* with a shuttle vector comprising:

(i) a mobilization region which permits transfer of the shuttle vector from *Escherichia coli* to a colonic Bacteroides species;

(ii) a mobilization region which permits transfer of the shuttle vector from the colonic Bacteroides species to the *P. ruminicola:* and

(iii) the heterologous DNA operatively linked to a promoter functional in the *P. ruminicola:*

(b) contacting the *E. coli* with a colonic Bacteroides species under conditions sufficient so that the shuttle vector is transferred from the *E. coli* to the colonic Bacteroides species; and

(c) contacting the colonic Bacteroides species with the *P. ruminicola* under conditions sufficient so that the shuttle vector is transferred from the colonic Bacteroides species to the *P. ruminicola.*

2. The method of claim 1 wherein the colonic Bacteroides species contains the Tc^rEm^r 12256 element.

3. The method of claim 1 wherein the colonic Bacteroides species is *Bacteroides uniformis.*

4. The method of claim 1 wherein the shuttle vector is pRDB5.

5. The method of claim 1 wherein the *P. ruminicola* is *P. ruminicola* B_14.

6. The method of claim 1 wherein the promoter comprises the following sequence (SEQ ID NO:1):

```
AAAAATCCTC CTACTTTTGT TAGATATATT TTTTTGTGTA ATTTTGTAAT  50
CGTTATGCGG CAGTAATAAT ATACATATTA ATACGAGTTA TTAATCCTGT 100
AGTTCTCATA TGCTACGAGG AGGTATTAAA AGGTGCGTTT CGACAATGCA 150
TCTATTGTAG TATATTATTG CTTAATCCAA                       180.
```

7. The *P. ruminicola* produced by the method of any one of claims 1–6.

8. A shuttle vector comprising:

a mobilization region which permits transfer of the shuttle vector from *Escherichia coli* to a colonic Bacteroides species;

a mobilization region which permits transfer of the shuttle vector from the colonic Bacteroides species to a *Prevotella ruminicola;* and

heterologous DNA operatively linked to a promoter functional in *P. ruminicola.*

9. The shuttle vector of claim 8 which is pRDB5.

10. The shuttle vector of claim 8 wherein the promoter comprises the following sequence (SEQ ID NO:1):

```
AAAAATCCTC CTACTTTTGT TAGATATATT TTTTTGTGTA ATTTTGTAAT  50
CGTTATGCGG CAGTAATAAT ATACATATTA ATACGAGTTA TTAATCCTGT 100
AGTTCTCATA TGCTACGAGG AGGTATTAAA AGGTGCGTTT CGACAATGCA 150
TCTATTGTAG TATATTATTG CTTAATCCAA                       180.
```

11. *Prevotella ruminicola* containing the shuttle vector of claim 8, 9 or 10.

12. The *P. ruminicola* of claim 11 which is *P. ruminicola* B_14.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,322,784
DATED : June 21, 1994
INVENTOR(S) : Abigail A. Salyers et al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 1, line 31, after "Bacteroides" insert --species.--.

Column 3, line 18, after "desirable" insert --.--.

Column 4, line 4, after "*ruminicola*" delete ":" and insert --;--.

Column 5, line 23, delete "transcription. translation" and substitute --transcription-translation--.

Column 5, line 58, delete "tetQ" and "tetQ" and substitute --*tetQ*-- and --*tetQ*--.

Column 5, line 59, delete "tetM" and substitute --*tetM*--.

Column 5, line 61, delete "tetO" and substitute --*tetO*--.

Column 5, line 64, delete "tetM" and substitute --*tetM*--; delete "tetO" and substitute --*tetO*--.

Column 8, line 42, delete "Tc^rEnm^r" and substitute --Tc^rEm^r--.

Column 8, line 68, delete "sections" and substitute --selections--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,322,784
DATED : June 21, 1994
INVENTOR(S) : Abigail A. Salyers et al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 10, line 14, delete second "A".

Column 10, line 26, delete "TN4351" and substitute --TN4351--.

Column 10, line 27, delete "626.632" and substitue --626-632--.

Column 10, line 47, delete "TN4351" and substitute --TN4351--.

Column 10, line 56, delete "TN1000" and substitute --TN1000--.

Column 10, line 59, delete "TN1000" and substitute --TN1000--.

Column 10, line 60, delete "TN1000" and substitute --TN1000--.

Column 10, line 64, delete "TN1000" and substitute --TN1000--.

Column 10, line 65, delete "TN1000" and substitute --TN1000--.

Column 11, line 6, delete "13.2" and substitute --13-2--.

Column 14, line 2, delete "rifampioin" and substitute --rifampicin--.

Column 15, line 16, delete "TcEm^r" and substitute --Tc^{r}Em^r--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,322,784

DATED : June 21, 1994

INVENTOR(S) : Abigail A. Salyers et al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 16, line 33, delete "n" and substitute --in--.

Column 16, line 42, delete "TetM" and substitute --TetM--; delete "TetO" and substitute --TetO--.

Column 16, line 46, delete "TetM" and substitute --TetM--; delete "TetO" and substitute --TetO--.

Column 16, line 61, delete "2537" and substitute --3537--.

Column 18, line 41, delete "13.2" and substitute --13-2--.

Column 18, line 50, after "kit" insert --(U.S.--.

Column 19, line 3, delete "lac" and substitute --*lac*--.

Column 19, line 26, delete "lac" and substitute --*lac*--.

Column 20, line 16, delete "Tc^r" and substitute --Tc^s--.

Column 20, line 18, delete "Tc^r" and substitute --Tc^s--.

Column 20, line 22, delete "transcription. translation" and substitute --transcription-translation--.

Column 22, line 8, delete "13.2" and substitute --13-2--.

Column 22, line 12, delete "13.2" and substitute --13-2--.

Column 22, line 25, delete "tetM" and substitute --tetM--; delete "tetO" and substitute --tetO--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,322,784
DATED : June 21, 1994
INVENTOR(S) : Abigail A. Salyers et al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 22, line 29, delete "tetM/O" and substitute --tetM/O--.

Column 22, line 30, delete "tetQ" and substitute --tetQ--.

Column 22, line 31, delete "tetQ" and substitute --tetQ--.

Column 23, line 12, delete "tetM" and substitute --tetM--; delete "tetO" and substitute --tetO--.

Column 23, line 38, delete "tetM" and substitute --tetM--; delete "tetO" and substitute --tetO--.

Column 23, line 41, delete "tetM" and substitute --tetM--; delete "tetO" and substitute --tetO--.

Column 23, line 46, delete "tetO" and substitute --tetQ--.

Column 23, line 55, delete "argF-lac" and substitute --argFlac--.

Column 23, line 56, delete "lac" and substitute --lac--.

Column 24, line 47, delete "M5697" and substitute --M25697--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,322,784
DATED : June 21, 1994
INVENTOR(S) : Abigail A. Salyers et al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

IN THE CLAIMS

Col. 51, Claim 1, line 10, delete ":" and substitute --;--.
Claim 1, line 12, delete ":" and substitute --;--.
Col. 52, Claim 6, line 6, at the end of the sequence insert --.--
Claim 10, line 7, at the end of the sequence insert --.--

Signed and Sealed this

Seventeenth Day of September, 1996

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks