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Construction of mutant strains of *Neisseria gonorrhoeae* lacking new antibiotic resistance markers using a two gene cassette with positive and negative selection

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Abstract

The pathogenesis of infections caused by *Neisseria gonorrhoeae*, the causative agent of the sexually transmitted disease gonorrhea, can be studied using experimental infection of human male volunteers. The desire to avoid introducing new antibiotic resistance markers into strains to be used in human experimental infection has complicated the construction of genetically defined mutants in which expression of potential virulence factors is inactivated. To facilitate construction of such mutants, we have used a two-step mutagenesis strategy that allows for gene replacements without introducing new selectable markers into the final strain. The method uses a two-gene cassette containing both a selectable marker (*ermC′*) and a counterselectable marker (*rpsL*). The cassette is cloned into the gene of interest and used to replace the wild-type gene on the chromosome by allelic exchange. A second transformation replaces the cassette-containing version of the gene with an engineered version with an unmarked deletion or other mutation. The *rpsL* gene of *Escherichia coli* functioned for the counterselection in the gonococcus, albeit with low efficiency. To improve the efficiency of the counterselection, we cloned the gonococcal *rpsL* gene and incorporated it into the cassette. This technique has been successful in creating defined mutants for human challenge, and also circumvents the limitation in the number of different selectable markers that are useful in *Neisseria* species. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Gonococcus; Human experimental infection; *rpsL* counterselection; Transformation

1. Introduction

Neisseria gonorrhoeae, the etiological agent of the sexually transmitted disease gonorrhea, is an obligate human pathogen. Research on the molecular basis of gonococcal pathogenesis has been hampered by the lack of appropriate animal models of infection. To overcome this limitation, experimental infection of human male volunteers has been used to study gonococcal virulence factors in vivo (Swanson et al., 1987, 1988; Schneider et al., 1991; Jerse et al., 1994; Seifert et al., 1994; Cornelissen et al., 1998). Experimental infection of men is safe and closely mimics natural infection [reviewed in Cohen et al. (1994)]. One use of the model is determining the importance of various components of the organism in pathogenesis by assaying infectivity of genetically defined mutants that do not express the gene of interest. However, constructing such mutants is complicated by the need to avoid introducing new antibiotic resistance markers into strains to be tested in humans. Although gonococci are naturally competent for DNA transformation, detection of desired transformants can be difficult without the use of a selectable marker. Gunn and Stein (1996) developed an efficient non-selective transformation technique in which a limiting number of gonococci are incubated with an excess amount of DNA and plated on non-selective medium; a substantial percentage of the resulting colonies contain cells that have been transformed. As an additional approach to engineering genetically defined gonococcal mutants without introducing new selectable markers in the final strain, we

Abbreviations: *E.*, *Escherichia*; Erm, erythromycin; Erm^R, erythromycin resistant; Erm^S, erythromycin sensitive; *N.*, *Neisseria*; oligo, oligodeoxyribonucleotide; PCR, polymerase chain reaction; Sm, streptomycin; Sm^R, streptomycin resistant; Sm^S, streptomycin sensitive; $rpsL_{ec}$, *rpsL* gene from *E. coli*; $rpsL_{gc}$, *rpsL* gene from *N. gonorrhoeae*.

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have used a two-step mutagenesis strategy with a two-gene cassette containing a selectable and a counter-selectable marker.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

N. gonorrhoeae strains FA1090 (Cohen et al., 1994) and F62 are Sm^R and Sm^S clinical isolates respectively. Gonococcal strains were maintained on GCB agar (Difco) containing supplements (Kellogg et al., 1968) at 37°C with 5% CO_2. Piliated colonies were chosen under a dissecting microscope based on colony morphology (Kellogg et al., 1968). Antibiotic concentrations for *N. gonorrhoeae* were: Erm, 5 μg/ml; Sm, 750 μg/ml or 10.5 mg/ml. *E. coli* DH5αMCR (Bethesda Research Laboratories) was used as a host strain for all recombinant plasmid constructions, and was maintained on LB-agar (Sambrook et al., 1989). *E. coli* HB101 (*rpsL*20) is Sm^R (Sambrook et al., 1989). Plasmid pFLOB 4200 contains the *opaK* gene of gonococcal strain FA1090 cloned into pBR322 (unpublished data). Antibiotic concentrations for *E. coli* were: Erm, 350 μg/ml; ampicillin, 100 μg/ml; Sm, 500 μg/ml. All antibiotics were purchased from Sigma.

2.2. Cloning techniques and PCR

Restriction enzymes were purchased from New England Biolabs and Boehringer Mannheim and were used according to the manufacturers' instructions. Vectors were treated with HK Phosphatase (Epicentre Technologies) prior to ligation with T4 ligase (Boehringer Mannheim).

To construct the *ermC′–rpsL*$_{ec}$ cassette, the *ermC′* gene (1.2 kb) and the *rpsL*$_{ec}$ gene (0.6 kb) were cloned into restriction sites located 23 bp apart in the *opaK* gene from gonococcal strain FA1090. The *ermC′* gene (Projan et al., 1987) was previously cloned between the *Hin*dIII and *Cla*I sites of pUC18 to yield plasmid pUC–ErmC′ (D. Trees and J.G. Cannon, unpublished data). The *Cla*I–*Hin*dIII fragment containing *ermC′* was isolated, blunt ended with T4 polymerase, and cloned into the unique *Kpn*I site (also blunt-ended) in the HV1 region of the cloned *opaK* gene. The amplimers 5′ GACGATAGTCATGCCCCGCGGCCGCCGGA and 5′ CATGCACCATTCCTTGCGGCCGCGGTGC were used to amplify *rpsL*$_{ec}$ with its native promoter from pSS1311 (Stibitz et al., 1986) using the following profile: denature at 94°C for 1 min, anneal at 50°C for 1.5 min, and extend at 72°C for 2 min for 30 cycles. The amplimers were complementary to pBR322 sequences flanking either side of the unique *Sal*I site of pSS1311 and contained engineered *Not*I sites (underlined). The PCR product was digested with *Not*I, purified on a Wizard PCR Purification column (Promega), and cloned into the unique *Not*I site in *opaK*. The 10 bp uptake sequence that is necessary for transformation of *Neisseria* species (Goodman and Scocca, 1988; Elkins et al., 1991) was cloned into the *Hin*dIII site at the 5′ end of *opaK*. Complementary oligos Upt1 (5′-GGGCAAGCTTG-CCGTCTGAAAAGCTTGGGC-3′) and Upt2 (5′-GCCCAAGCTTTTCAGACGGCAAGCTTGCCC-3′) (100 pmol of each) were boiled for 5 min then annealed by slow cooling to room temperature. The annealed oligos, containing one copy of the gonococcal uptake sequence flanked by *Hin*dIII sites (underlined), were digested with *Hin*dIII and cloned into the *Hin*dIII site of the plasmid, which was designated pFLOB 4250. The construct was confirmed by DNA sequencing, as were all similar constructs, performed by the UNC-CH Automated DNA Sequencing Facility on a Model 373A DNA Sequencer (Applied Biosystems) using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems).

Plasmid pFLOB 4260, containing the *opaK* gene inactivated by a linker insertion, was constructed by annealing complementary oligos Inact 1 (5′-CC-GGTACCGCCTTAATTAATTTAGCCGGTACCGGG) and Inact 2 (5′-CCCGGTACCGGCTAAATTAATT-AAGGCGGTACCGG) as described above. The annealed oligos, containing termination codons in all three translational frames and an internal *Pac*I site flanked by *Kpn*I sites (underlined), were digested with *Kpn*I and cloned into the *Kpn*I site of the *opaK* gene in pBR322. This clone, designated pFLOB 4260, also contained the uptake sequence 5′ of *opaK*.

To construct the *ermC′–rpsL*$_{gc}$ cassette, the *rpsL* gene from gonococcal strain F62 was amplified with its native promoter from chromosomal DNA using the amplimers gcrpsL1 (5′ GGCGAATTCCCGACTGATTGTGA-GGGATGTCGG) and gcrpsL2 (5′ CTGGGATCC-GTTGTTCAGCTTAGGCGGCCGACG), which were based on the strain FA1090 sequence from the Gonococcal Genome Sequencing Project (Roe et al., 1999), and which contained *Eco*RI and *Bam*HI sites respectively (underlined). The primers were used for PCR using the following profile: denature at 94°C for 1 min, anneal at 50°C for 1 min, and extend at 72°C for 1 min for 30 cycles. The resulting 568 bp PCR product was digested with *Eco*RI + *Bam*HI, purified, and cloned into the corresponding sites in the polylinker of pUC–ErmC′, resulting in plasmid pFLOB 4300. The *ermC′–rpsL*$_{gc}$ cassette was introduced into the cloned *opaK* gene by amplifying the 1.8 kb cassette using pUC Forward and Reverse primers in a PCR reaction as above, and cloning it into the *Kpn*I site (blunt-ended) in *opaK*.

All plasmids containing *rpsL* from either *E. coli* or *N. gonorrhoeae* were transformed into Sm^R *E. coli* strain

HB101 (*rpsL*20), to confirm that the cloned copy of *rpsL* conferred dominant Sm sensitivity when present in combination with the chromosomal Sm^R allele. However, the *rpsL*-containing plasmids were unstable in the Sm^R host strain, which was therefore not used for routine plasmid maintenance.

2.3. Bacterial DNA transformations

Gonococcal transformations were performed using a modification of the protocol of Gunn and Stein (1996) as follows: piliated gonococci were identified by colony morphology and streaked for isolated colonies on GCB agar plates. Transforming DNA was prepared using bacterial chromosomal DNA isolation columns (Qiagen) and diluted to 0.2 mg/ml in $1 \times$SSC with 1 mM $MgCl_2$. The diluted DNA was spotted on marked areas of the plates in 10 μl drops and allowed to absorb; plates were then incubated overnight at 37°C with 5% CO_2. The following day, growth within the marked areas was collected on a cotton swab, suspended in GCB broth, diluted and plated on GCB agar plates containing the appropriate antibiotic. *E. coli* strains were made competent with DMSO and were transformed with ligations following standard protocols (Sambrook et al., 1989).

3. Results and discussion

3.1. Mutagenesis strategy

The strategy involves using a two-gene cassette containing a selectable marker (*ermC′*, conferring Erm resistance) and a counterselectable marker (*rpsL*, cloned from an Sm^S strain). The aminoglycoside antibiotic Sm inhibits protein synthesis in susceptible cells by binding to the small ribosomal subunit. Sm^R organisms often have a mutation within the *rpsL* gene encoding ribosomal protein S12 (Sparling et al., 1968). Merodiploid organisms containing one sensitive allele and one resistant allele are phenotypically Sm^S, because sensitivity is dominant over resistance (Lederberg, 1951; Breckenridge and Gorini, 1970). Thus, when the sensitive allele of *rpsL* is introduced into the chromosome of an Sm^R strain as a merodiploid, the resulting strain is Sm^S. Counterselection, in which direct selection is applied for loss of the Sm^S allele, is accomplished by plating the organisms on Sm. This phenomenon has been exploited in several bacteria for plasmid curing, suicide vectors, and gene replacements [reviewed in Reyrat et al. (1998)].

In the modification of the counterselection strategy that we have used (outlined in Fig. 1), the first necessary construct contains the two-gene cassette inserted into the cloned gene of interest. This suicide plasmid is used to transform an Sm^R recipient gonococcal strain, selecting for the Erm resistance marker and screening for Sm sensitivity. The second construct consists of the cloned gene of interest inactivated by an unmarked mutation such as a small insertion or deletion, which is used to transform one of the Erm^R Sm^S transformants from the first transformation. Sm^R transformants are those in which the cassette-containing copy of the gene on the chromosome has been replaced by the copy with the unmarked mutation. Thus, by sequentially selecting for the gain and subsequent loss of the *ermC′–rpsL* cassette, the gene of interest is inactivated without adding any new antibiotic resistance markers. This general strategy has been used previously in other bacteria, including *Bordetella pertussis* (Zealey et al., 1990).

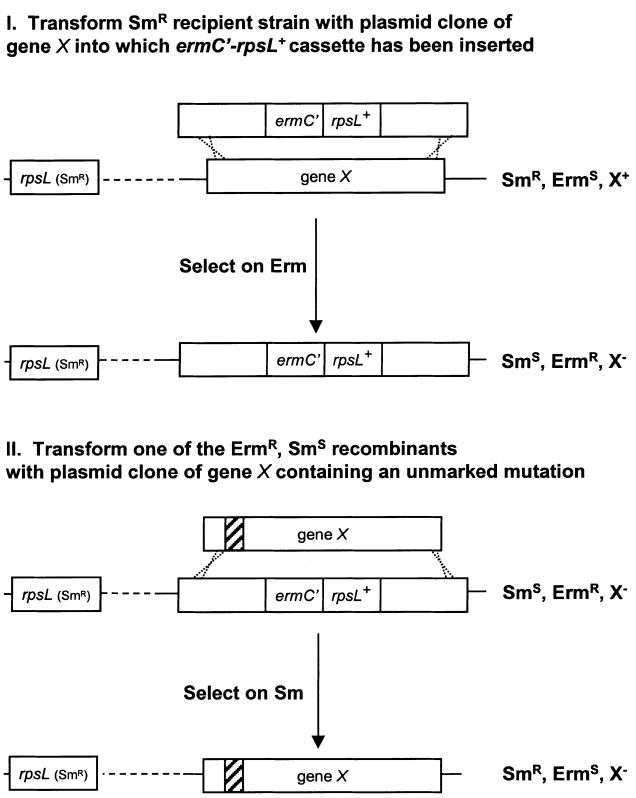

Fig. 1. Mutagenesis strategy using *ermC′–rpsL*. The *ermC′–rpsL* cassette is cloned into the gene of interest on a plasmid clone, using a vector that does not replicate in *Neisseria*. This construct is used to transform an Sm^R gonococcal strain, selecting for Erm^R transformants in which the wild-type gene has been replaced by the engineered version containing the cassette. One of these transformants, which is Sm^S by virtue of the presence of $rpsL^+$, is used as a recipient in the second transformation, for which the donor DNA is a second plasmid clone with an unmarked alteration in the gene of interest. Final transformants in which the unmarked version of the inactivated gene has replaced the cassette-containing gene on the chromosome are selected on Sm and screened for Erm sensitivity.

3.2. Use of the E. coli rpsL gene for counterselection

We tested the strategy by constructing an unmarked mutation in the *opaK* gene of Sm^R gonococcal strain

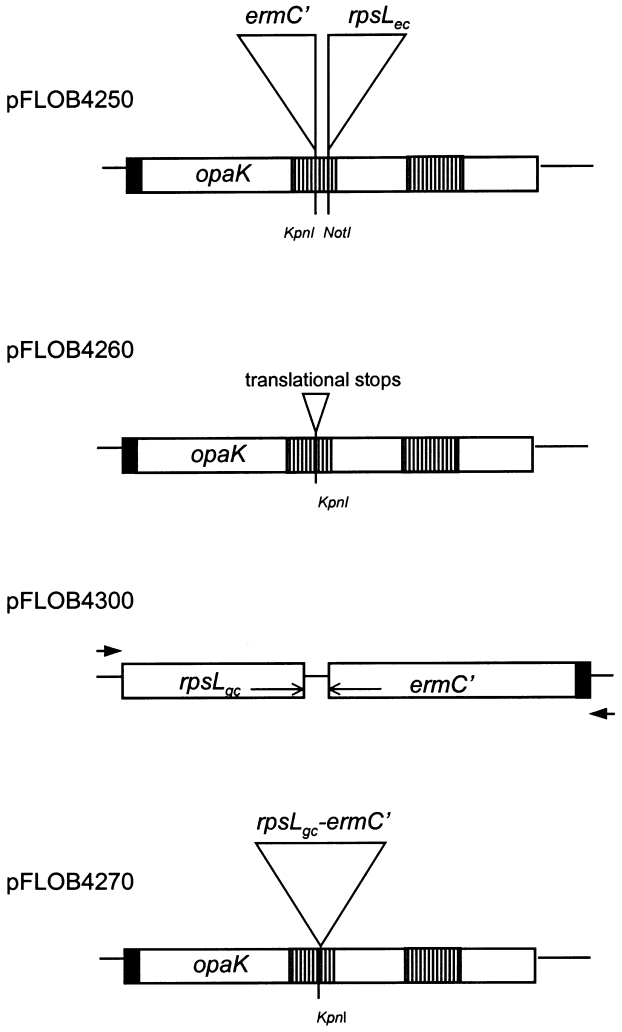

Fig. 2. Plasmid constructs used in constructing mutant strains of *N. gonorrhoeae*. pFLOB 4250 was made by cloning the *ermC′* and *rpsL*$_{ec}$ genes into unique restriction sites located 23 bp apart in the *opaK* gene in plasmid pBR322. The 10 bp neisserial DNA uptake sequence (solid black boxes) was cloned into the *Hin*dIII site at the 5′ end of the *opaK* gene (pFLOB4250, 4260, and 4270) or the *ermC′* gene (pFLOB4300). Plasmid pFLOB 4260 contains a 24 bp linker insertion encoding translational terminators in three frames; the insertion also contains a *Pac*I site. The shaded regions represent the hypervariable regions (regions differing in sequence among different *opa* genes) of *opaK*. Plasmid pFLOB4300 contains *ermC′* and *rpsL*$_{gc}$ cloned into the polylinker of plasmid pUC18. The orientation of the two genes is shown by the arrows within the boxes corresponding to the genes. The *ermC′–rpsL*$_{gc}$ cassette was amplified using pUC Forward and Reverse primers (designated by filled arrowheads) in a PCR reaction, and was cloned into the *Kpn*I site of *opaK*, forming plasmid pFLOB 4270. Diagrams are not drawn to scale.

FA1090. The *opaK* gene is one of a family of genes encoding antigenically distinct Opacity (Opa) proteins, which are surface proteins that are variably expressed during human infection (Swanson et al., 1988; Jerse et al., 1994). The two required constructs were made using a cloned *opaK* gene in plasmid pBR322, which does not replicate in *Neisseria* species (Fig. 2). Fragments containing the *ermC′* gene and *rpsL*$_{ec}$ were ligated into restriction sites in *opaK* that are spaced 23 bp apart, resulting in plasmid pFLOB 4250. Both of the inserted genes contained their own promoters (Stibitz et al., 1986; Projan et al., 1987). The second construct consisted of a small linker insertion containing translational terminators in all three frames that was cloned into a unique restriction site in *opaK*. The 10 bp neisserial uptake sequence that is necessary for DNA transformation of gonococci (Elkins et al., 1991) was cloned into a restriction site at one end of the *opaK* insert in both plasmids. We initially used *rpsL*$_{ec}$ in the hope that it would be expressed efficiently in *Neisseria* species and would confer an Sm^S phenotype upon the Sm^R strain FA1090 when present in a single copy on the chromosome.

Strain FA1090 (Sm^R) was transformed with pFLOB 4250 (*opaK::ermC′–rpsL*$_{ec}$), selecting for Erm^R transformants. The Erm^R transformants showed no difference from the Sm^R parent strain in growth on the concentration of Sm that is usually used to distinguish Sm^R and Sm^S strains of *N. gonorrhoeae* (200 μg/ml). However, the transformants containing *rpsL*$_{ec}$ grew noticeably slower at high concentrations of Sm, forming smaller colonies than FA1090 after incubation for 16–18 h on agar medium containing >10 mg/ml Sm. We exploited this difference in growth rates to enrich for Sm^R and Erm^S transformants in the second transformation by multiple transfers of the transformation mixture onto medium containing 10.5 mg/ml of Sm, with short (16–18 h) incubation times. Plasmid pFLOB 4260 (*opaK*::linker insertion) was used to transform one of the transformants with the *ermC′–rpsL*$_{ec}$ cassette in *opaK* that had resulted from the first transformation step. After three cycles of Sm enrichment, approximately 20% of the colonies tested were Erm^S. Southern blotting experiments demonstrated that the allelic replacement in both the intermediate and final transformants had occurred as expected, and that the chromosomal *opaK* gene contained the inactivating linker insertion (data not shown). Subsequent analysis for Opa protein expression confirmed that the mutation did prevent expression of the protein (data not shown). The antibiotic resistance phenotype of the final mutant strain was identical to that of the original parent strain (Sm^R, Erm^S).

3.3. Cloning of the gonococcal rpsL and its use in counterselection

To improve the efficiency of the *rpsL* counterselection, we took advantage of newly available sequence data from the Gonococcal Genome Sequencing Project (Roe et al., 1999) to clone the wild-type gonococcal *rpsL* gene and incorporate it into the *ermC′–rpsL* cassette in place of the *E. coli* gene. We identified a probable *rpsL* gene in the sequence database through homology to pre-

viously cloned *rpsL* genes from other bacterial species (Stibitz, 1994; Sander et al., 1995; Skorupski and Taylor, 1996; Hosted and Baltz, 1997). Although the database sequence is that of Sm^R strain FA1090, we assumed that the flanking sequence would be similar in Sm^S strains and designed PCR primers based on the FA1090 sequence that would amplify the entire *rpsL* gene, including the probable promoter and transcriptional terminator. We used the primers in PCR with DNA from Sm^S strain F62 as template and cloned the amplified product adjacent to *ermC′* in the polylinker of plasmid pUC18–ErmC′, resulting in plasmid pFLOB4300.

To test the ability of the $rpsL_{gc}$-containing cassette to confer Sm sensitivity upon an Sm^R gonococcal strain, we amplified $rpsL_{gc}$–*ermC′* from pFLOB4300 and inserted the cassette into the cloned *opaK* gene, resulting in plasmid pFLOB 4270. When pFLOB 4270 was used to transform FA1090 to Erm^R, the Erm^R transformants were sensitive to 750 μg/ml of Sm. Replacement of the cassette on the chromosome by transformation with pFLOB 4260 (*opaK*::linker insertion) was readily accomplished by selecting transformants at this level of Sm. The Sm^R transformants were Erm^S, with the *opaK* gene inactivated by the linker insertion. The gonococcal $rpsL_{gc}$ gene also conferred sensitivity to 750 μg/ml Sm when introduced as a merodiploid into an Sm^R strain of *N. meningitidis* (data not shown), indicating that the method will be useful for making mutants of the meningococcus.

The success of the counterselection with both $rpsL_{ec}$ and $rpsL_{gc}$ confirmed that the mechanism of streptomycin resistance in gonococcal strain FA1090 involves a mutational alteration in ribosomal protein S12, and also confirmed the identity of the cloned F62 gene as *rpsL*. The partial dominance of $rpsL_{ec}$ in the Sm^S/Sm^R merodiploids could have been due to poor expression of the *E. coli* gene in the gonococcal host strain, or to partial defects in assembly or function of gonococcal ribosomes containing the *E. coli* S12 ribosomal protein.

Recovery of transformants in each of the steps of the procedure (using the constructs containing either $rpsL_{ec}$ or $rpsL_{gc}$) was greatly enhanced by using the agar plate transformation method derived from that of Gunn and Stein (1996). Transformants that were difficult or impossible to recover using a classical broth transformation technique were readily obtained using the agar plate transformation method, presumably because the transformation efficiency was higher (data not shown). However, a disadvantage of the agar plate transformation method is that we could not determine exact transformation frequencies using the different constructs.

We have used this mutagenesis strategy to construct mutant strains that have been tested in the human challenge model (Cornelissen et al., 1998; Johannsen et al., 1999). In each case, we amplified the cassette with the desired restriction sites incorporated into the PCR primers; the amplified cassette was then cloned into the gene of interest. The Sm^R phenotype of the challenge strains does not alter their susceptibility to ciprofloxacin or ceftriaxone, the antibiotics that are used to cure the experimental infections. Another application of the method is the sequential mutagenesis of multiple genes in one strain, thus circumventing the limitations in the number of different selectable markers that are useful in *Neisseria* species. The necessity for engineering two constructs in order to use the *rpsL* counterselection strategy is an inconvenience. However, this method is particularly appealing despite the inconvenience when the engineered version of the gene to be recombined onto the chromosome has a large deletion or insertion (such as a large reporter gene). Under such circumstances, the recombination frequency will be low and recombinants may be difficult or even impossible to detect without selection. This method complements other currently available methods for engineering neisserial strains without introducing new selectable genetic markers (Gunn and Stein, 1996).

3.4. Conclusions

(1) The *rpsL* gene can be used as a counterselectable marker in *N. gonorrhoeae* (and *N. meningitidis*) as in other bacteria, even when present in a single copy on the chromosome.

(2) The *rpsL* genes of *E. coli* and *N. gonorrhoeae* encode functionally similar versions of ribosomal protein S12. However, the Sm^S allele of the *E. coli* gene showed only partial dominance when present as a merodiploid in an Sm^R gonococcal strain.

(3) Unmarked mutations can be readily introduced into the chromosome of Sm^R gonococcal strains using the two-step selection/counterselection method with the *ermC′*–$rpsL_{gc}$ cassette.

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