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An *rpsL* Cassette, Janus, for Gene Replacement through Negative Selection in *Streptococcus pneumoniae*

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Natural genetic transformation offers a direct route by which synthetic gene constructs can be placed into the single circular chromosome of *Streptococcus pneumoniae*. However, the lack of a general negative-selection marker has hampered the introduction of constructs that do not confer a selectable phenotype. A 1.3-kb cassette was constructed comprising a kanamycin (Kn) resistance marker (*kan*) and a counterselectable *rpsL*$^+$ marker. The cassette conferred dominant streptomycin (Sm) sensitivity in an Sm-resistant background in *S. pneumoniae*. It was demonstrated that it could be used in a two-step transformation procedure to place DNA of arbitrary sequence at a chosen target site. The first transformation into an Sm-resistant strain used the cassette to tag a target gene on the chromosome by homologous recombination while conferring Kn resistance but Sm sensitivity on the recombinant. Replacement of the cassette by an arbitrary segment of DNA during a second transformation restored Sm resistance (and Kn sensitivity), allowing construction of silent mutations and deletions or other gene replacements which lack a selectable phenotype. It was also shown that gene conversion occurred between the two *rpsL* alleles in a process that depended on *recA* and that was susceptible to correction by mismatch repair.

Streptococcus pneumoniae, a widespread human pathogen associated with high rates of disease and mortality, is being used increasingly as a genetically tractable model pathogen for application of genomics to searches for new drugs and drug targets. Many wild-type strains of the pneumococcus are readily transformable (18), so that effects of disruption of specific genes can be readily assessed in model host systems. To date, these gene disruptions have commonly been made by inserting a drug resistance gene that provides direct selection of rare recombinants. While powerful, this method does have drawbacks. As design of strains with multiple mutations becomes more sophisticated, for example, an accumulation of drug markers in the mutated strains could become cumbersome and possibly compromise interpretations of experimental results. Also, many important categories of gene mutation, such as missense substitutions and in-frame deletions, usually confer no selectable phenotype.

While there are several currently available ways to circumvent or accommodate these limitations, a particularly convenient approach, used in a variety of other bacteria but not yet applied in *S. pneumoniae*, employs a bicistronic cassette permitting both selection for its acquisition and selection for its loss. One marker allowing the required negative selection is based on a common spontaneous bacterial streptomycin (Sm) resistance mutation in the gene *rpsL* that causes a lysine replacement in protein S12 of the small ribosomal subunit (21). As this mutation is recessive, an *rpsL*$^+$ allele has been employed to provide a dominant drug-sensitive phenotype in genetic contexts where it can provide direct selection for deletion, mutation, or replacement events (5, 7, 8, 17, 20, 22–25). Here we describe application of this principle for construction of an *rpsL* cassette for use in *S. pneumoniae* that allows use of antibiotics at both selection steps and show that it can be used with natural genetic transformation for gene replacement through negative selection.

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MATERIALS AND METHODS

Strains, media, and DNA sources. *S. pneumoniae* strains used in this work (Table 1) are derivatives of strains R6 and Rx, whose origins are traced by Tiraby et al. (26). The recessive *str41* mutation (4) corresponds to a single base substitution (A to C transversion) converting Lys56 (AAA) to Thr (ACA) (21); the allele *str1* confers the same K56T substitution on S12 but also carries two silent transitions (GC to AT), at positions 150 and 405 in the *rpsL* gene (13). We refer to these Sm^r alleles here as *rpsL41* and *rpsL1*, respectively. Growth media and culture methods for genetic transformation have been described (10, 12). Unless stated otherwise, donor DNA was used at a final concentration of 100 ng/ml of competent culture. Drug selection was as described (10) using final concentrations in selective agar of 150 μg of Sm per ml, 200 μg of kanamycin (Kn) per ml, and 2.5 μg of novobiocin (Nv) per ml. For transformation, cultures growing in broth were induced with synthetic competence-stimulating pheromone peptide (CSP-1; Chiron Mimitopes, Raleigh, N.C.) as described (6).

PCR amplification. Plasmid or genomic DNA (50 ng) and 50 pmol of each primer were used in a total volume of 50 μl of PCR SuperMix (Gibco-BRL); amplification proceeded for 30 cycles as follows: 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C, followed by a 10-min extension cycle. Products were purified using the QIAquick PCR purification kit (Qiagen). Oligonucleotide primers used for various purposes are listed in Table 1.

Construction of a positive/negative selection cassette, *kan-rpsL*$^+$. A 435-bp fragment (PCRII) containing *rpsL*$^+$ was amplified from chromosomal DNA of strain R800 (*Eco*RI and *Apa*I termini) using the primer pair DAM350 and DAM351. A 896-bp fragment (PCRIII) containing the *kan* marker was amplified from plasmid pR410 using DAM301 and DAM347 (*Bam*HI and *Eco*RI termini). Plasmid pR410 (kindly provided by Marc Prudhommme) carries a synthetic *kan* cassette derived from the *aphIII* gene of plasmid pJHI (27) and was designed similarly to the previously described erythromycin and chloramphenicol resistance cassettes (1). Two DNA fragments (PCRI and PCRIV) flanking the dispensable target gene *cbp3* (19) were prepared by PCR using chromosomal DNA of strain CP1250 as a template (PCRI, with a *Bam*HI 3′ terminus

TABLE 1. Bacterial strains and primers used in this study

Strain or primer	Description	Source or reference
S. pneumoniae		
CP1200	Rx derivative; *hex mal rpsL1*; Hex$^+$ Kn^s RecA$^+$ Sm^r	14
CP1250	CP1200 but *bgl-1*; Kn^s Sm^r	16
CP1296	CP1250 but *cbp3*::*kan-rpsL*$^+$ (by transformation with PCR construct; see Materials and Methods); Kn^r Sm^s	This study
CP1326	CP1296 but *rpsL*$^{+/+}$ by spontaneous conversion; *cbp3*::*kan-rpsL*$^+$; Kn^r Sm^s	This study
R800	R6 derivative; Hex$^+$ Kn^s RecA$^+$ Sm^s	11
R239	R800 but *recA*::*ermAM* (by transformation with a ligation mixture)-deletion *Cla*I (*recA*)-*Cla*I (within RUP, downstream of *recA*), blunt ended and ligated to a *Bst*UI-*ermAM* fragment; Ery^r	Bernard Martin
R304	R800 but *nov1 rif23 rpsL41*; Nov^r Rif^r Sm^r	15
R416	R800 but *rpsL41* (by transformation with R304 chromosomal DNA); Sm^r	This study
R810	R800 but *comC*::pXF520 (*comC*$^+$) *ebg*::*spc*, and carries UP mutation; ComCDE^{UP} Cm^r Spc^r	12
R960	R416 but *cbp3*::*kan-rpsL*$^+$ (by transformation with CP1296 chromosomal DNA); Hex$^+$ *rpsL41* Kn^r Sm^s	This study
R961	R800 but *cbp3*::*kan-rpsL*$^+$ (by transformation with CP1296 chromosomal DNA); Hex$^+$ Kn^r	This study
R974	R960 but Sm^r through spontaneous conversion; *cbp3*::*kan-rpsL41 rpsL41*; Kn^r Sm^r	This study
R981	R800 but *rpsL1* (by transformation with CP1200 chromosomal DNA); Sm^r	This study
R989	R960 but *recA*::*ermAM* (by transformation with R239 chromosomal DNA); *cbp3*::*kan-rpsL*$^+$ Hex$^+$ *rpsL41*; Ery^r Kn^r Sm^s	This study
R990	R981 but *cbp3*::*kan-rpsL*$^+$ (by transformation with CP1296 chromosomal DNA); *rpsL1*; Kn^r Sm^s	This study
R1001	R989 but spontaneous revertant to Sm^r; *cbp3*::*kan-rpsL*^{0} *recA*::*ermAM rpsL41*; Ery^r Kn^r Sm^r	This study
R1004	R960 but *hexA*::*cat*; Cm^r Kn^r Sm^s	This study
R1005	R990 but *hexA*::*cat*; Cm^r Kn^r Sm^s	This study
R1029	R800 but Δ*comC*::*kan-rpsL*$^+$ (by transformation with a SOEing fragment; see Materials and Methods); Kn^r	This study
R1036	R981 but Δ*comC*::*kan-rpsL*$^+$ (by transformation with R1029 chromosomal DNA); Kn^r Sm^s	This study
Primers[a]		
B	CATTATCCATTAAAAATCAAACGGAAGCCGGGAAAATTCCCAGC (underlined sequence corresponding to *kan* cassette; complementary to Kan5, see below); upstream of *comC*; 679–660; SPU33315	
BM47	GATTTGCTAAGTTTGAAATGATTGAG; within *orfL*, upstream of *comCDE*; 3–28; SPU33315	
BM52	GTCCTCTATCCCTCTCATAC; within *comD*; 1080–1061; SPU33315	
BM54	CATTCCAGCATAATCATGTCG; within *comD*; 1583–1563; SPU33315	
BM81	TCGCGATGACTACTATGAACG; upstream of *cbp3*; TIGR[b]	
BM82	GCTTACAGAAAAGAGCAGGAAAT; downstream of *cbp3*; TIGR	
C	GGAAAGGGGCCCAGGTCTCTGTAATGAAATAAGGGGAAAGAG (underlined sequence corresponding to *rpsL*; complementary to primer 7, see below); upstream of *comD*; 801–822; SPU33315	
cat3	AGCCAGTCATTAGGCCTATC; within *cat* (pC194 plasmid); 1903–1884; L08860	
DAM301	CGCGCAAGCTGGGGATCCG; upstream of *kan* in pR410	
DAM303	AAGGGCCCGTTTGATTTTTAATG; positions 7–23 of DAM303 correspond to positions 773–789 upstream of *kan*; AF411920; TIGR	
DAM313	AGCTTTCTCGTGGTGTAGAACAAC; upstream of *cbp3*; TIGR	
DAM314	ACGAGGATCCGATCCATTTCCTCTGGAATA; within *cbp3*; TIGR	
DAM315	AGCAGGGCCCAGGTCTCTGGTAAGTGGTAT; within *cbp3*; TIGR	
DAM316	CTCTCAAGGTCGCCCAGCTATG; downstream of *cbp3*; TIGR	
DAM345	CAGGAGACATTCCTTCCGTATCTT; within *kan*; 926–903; AF411920; TIGR	
DAM347	CCGAATTCTAGGTACTAAAACAATTCATCCAGTAA; downstream of Kan5 in pR410	
DAM350	CTGGAATTCACCAAAAATAAAAAAACACAG GAG; upstream of *rpsL*; TIGR	
DAM351	CTAGGGCCCCTTTCCTTATGCTTTTGGAC; downsteam of *rpsL*; TIGR	
Kan5	CCGTTTGATTTTTAATGGATAATG; positions 3–24 of Kan5 correspond to positions 185–206 of *ami*; X17337	
7	AGAGACCTGGGCCCCTTTCC; downstream of *rpsL*; TIGR	

[a] Information given in the order sequence; gene; position within the deposited sequence indicated; and GenBank/EMBL accession number.
[b] TIGR, TIGR website (http://www.tigr.org).

[DAM313-DAM314] and PCRIV, with an *Apa*I 5′ terminus [DAM315-DAM316]. PCRI, PCRII, PCRIII, and PCRIV were digested with corresponding restriction nucleases, purified, ligated, and used to transform CP1250, with selection for Kn^r. After backcrossing one Kn^r clone to CP1250, one of the resulting Kn^r transformants, named CP1296, was shown to carry a disruption of the target gene and insertion of the *kan* and *rpsL* fragments with the same polarity as *cbp3*. The predicted sequence of the disrupted *cbp3* locus is available (GenBank accession no. AF411920).

The structure of the insertion in CP1296 was verified by PCR with primer pairs DAM313-DAM347, DAM303-DAM351, and DAM350-DAM316; the expected junction fragments of 1.7, 1.3, and 1.2 kb, respectively, were obtained. The sequences of junctions were also verified by reading sequence in the junction fragments with primers DAM345, DAM351, and DAM350. The entire sequence within the *rpsL*$^+$ gene was read in both directions and matched the published sequence (Z15120). We propose the trivial name Janus for this cassette, which uses both forward and reverse selection to allow formation of junctions and new structures not under selection.

Construction of R1029, a Δ*comC*::*kan-rpsL*$^+$ strain. A strain harboring a substitution of the *comC* gene by the *kan-rpsL*$^+$ cassette was constructed as follows. First, a PCR fragment containing the region immediately upstream of

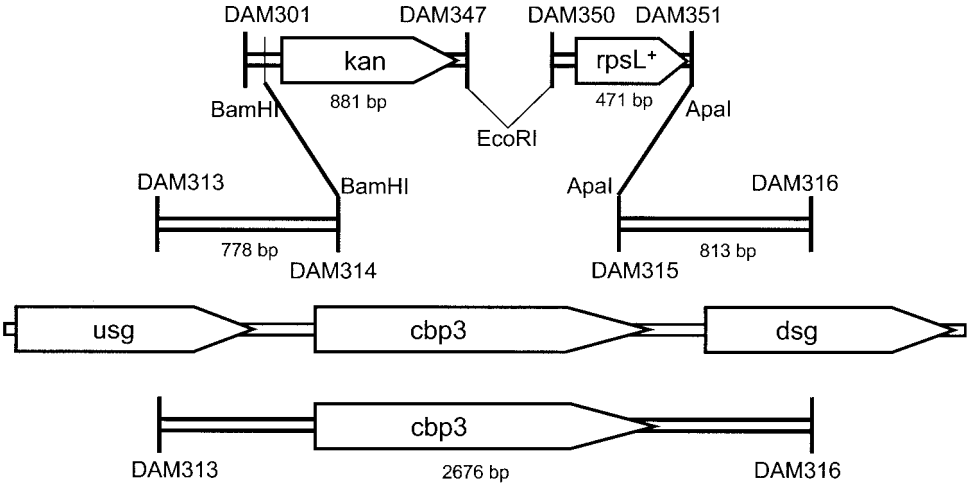

FIG. 1. Construction of Janus cassette in the *S. pneumoniae cbp3* locus. Pentagons marked rpsL and kan, modules of the Janus cassette. Primers used to amplify two cassette modules and two targeting fragments are indicated at the termini of those PCR products. After synthesis, restriction enzyme digestion, and purification, the product of a single ligation reaction was screened directly by transformation of CP1250 to obtain correctly linked modules giving the Kn^r phenotype. Top, PCR fragment used to construct strain CP1296 (*cbp3*::*kan-rpsL*$^+$). Middle, *cbp3* chromosomal region. Bottom, a 2,676-bp fragment containing *cbp3* amplified from chromosomal DNA of strain CP1250 with primers DAM313 and DAM316 and used for reintroduction of *cbp3* to replace the Janus cassette.

comC was amplified from R800 chromosomal DNA with the primers BM47 and B, and a *kan-rpsL* fragment was amplified from strain R960 with the Kan5 and 7 primers (Table 1). Following purification through QIAquick columns, the two fragments were mixed and connected to generate fragment A by PCR with the BM47-7 primer pair. A fragment containing part of *comD* was then amplified with the C-BM54 primer pair (Table 1), purified using a QIAquick column, and mixed with fragment A for assembly into a unique product through PCR amplification with primers BM47 and BM54. Strain R1029 (R800 Δ*comC*::*kan-rpsL*$^+$; Table 1) was obtained following transformation of strain R800 with the final PCR product by selection for Kn^r transformants. Normal transformant yields obtained with strain R1029 following treatment with synthetic competence pheromone (data not shown) indicated that the synthetic promoter in *kan-rpsL*$^+$ drove sufficient expression of *comDE* for pheromone signal transmission and full activation of the competence cascade.

RESULTS

Construction and properties of a dominant Sm^s cassette. A bicistronic positive/negative selection cassette was constructed by combining PCR amplicons containing a Kn resistance gene preceded by a constitutive promoter from the *amiA* (oligopeptide permease) locus (1) and the wild-type S12 gene, *rpsL*. The cassette was inserted into a dispensable gene coding for a putative choline-binding protein (*cbp3*) (19) in strain CP1250, which carries the *rpsL1* mutation in the "genuine" or chromosomal copy of *rpsL*, as described in Materials and Methods and illustrated in Fig. 1. PCR with characteristic sets of primers directed to the cassette and to the insertion locus confirmed that junction fragments and an internal *kan-rpsL* fragment were readily amplified from DNA of CP1296. The detailed organization of the cassette is displayed in AF411920, including the *rpsL* sequence determined for the copy of the gene obtained from R800 and its context in CP1296. Although CP1250 is Sm^r, CP1296 was found to be fully sensitive to this drug, as predicted for addition of a dominant Sm^s allele; the cassette reduced the Sm MIC from >250 to 5 μg/ml, the same as the MIC of R800, the Sm^s source of *rpsL*$^+$ (data not shown).

***kan-rpsL*$^+$ cassette can be deleted by targeted recombination.** Although Rimini et al. (19) reported transformation deficiency for an insertion-duplication mutation at *cbp3*, CP1296 was transformed as readily as its parent, CP1250. In contrast to a minority of spontaneous Sm^r revertants found in CP1296 cultures (see below), deliberate transformation of this strain with DNA from strain R800, which is Sm^s but carries an intact *cbp3* gene, generated a large number of Sm^r clones which had also become Kn^s (Table 2). In accord with the design of this cassette, we interpret these DNA-dependent recombinants as arising from excisional recombination exchange replacing the entire *kan-rpsL* cassette in a single event directed by flanking homology at the *cbp3* locus. As expected, transformation with a pure amplicon containing the *cbp3* gene (Fig. 1) was also effective at generating Kn^s Sm^r recombinants (data not shown). To verify this interpretation of the genetic results, several putative excisional transformants were examined by growing colonies from single Sm^r colonies; Kn^s clones were found only when a *cbp3*-containing donor DNA had been used for the cross (data not shown).

Replacement of the entire *kan-rpsL* cassette via transformation with a donor DNA lacking the cassette is thus readily

TABLE 2. Excisional transformation replaces *kan-rpsL* cassette with *cbp3*[a]

Donor DNA	Induction by CSP	Fold increase[b] in Sm^r	Growth (CFU/ml) with selection on:	
			Sm	Sm and Kn
None	+	1.1	1,000	830
	−	1	910	820
R800 (Sm^s)	+	28	22,000	1,000
	−	1	780	710

[a] Strain CP1296 was grown to an optical density of 0.05, diluted 10-fold, and treated as indicated with CSP-1 (100 ng/ml) and DNA (10 ng/ml) for 60 min. Cell types are reported among 10 to 20 million cells per ml in the transformed population.

[b] Ratio of Sm^r in CSP-treated culture to that in untreated control.

TABLE 3. Analysis of cotransfer of Kn^r and Sm^r to characterize spontaneous Sm^r revertants

Recipient strain	Donor DNA[a]	Growth (CFU/ml) with selection on:		Sm^r frequency per 100 Kn^r cells
		Kn	Sm	
R800	R974	2.99×10^6	3.6×10^4	1.20
	R989	4.18×10^6	<10	<0.0002
	R1001	2.86×10^6	<10	<0.0003
R416	R974	7.76×10^6	R[b]	
	R989	7.76×10^6	S[b]	
	R1001	7.76×10^6	R[b]	

[a] DNA from Kn^r transformant of R800 after transformation with the indicated strain.
[b] Cells from two Kn^r colonies were plated in the presence or absence of 200 μg of Sm per ml to assess their resistance (R) or susceptibility (S).

TABLE 4. Genuine → cassette gene conversion is RecA dependent

Strain	Genotype	Sm^r frequency per 10,000 cells[a]	Fold reduction[b]
R960[c]	*rpsL41 cbp3*::*kan-rpsL*$^+$ *hex*$^+$	0.47 ± 0.17	5.3
R989[c]	R960 but *recA*	0.012 ± 0.0057	207.5 (39.2[d])
R960	*rpsL41 cbp3*::*kan-rpsL*$^+$ *hex*$^+$	2.49 ± 0.62	1.0
R1004	R960 but *hexA*::*cat*	2.40 ± 0.63	1.0
R1005	R990 but *hexA*::*cat*	1.95 ± 0.93	1.3

[a] Calculated from four to five independent cultures grown from individual colonies resuspended in 2 ml of Todd-Hewitt broth supplemented with 0.5% yeast extract and grown to an OD_{550} of 0.5 to 0.7 before plating.
[b] Compared to the frequency of spontaneous conversion with the *rpsL41-rpsL*$^+$ combination (*hex*$^+$ background) for cultures grown in the presence of O_2 (last three lines; results taken from Table 5).
[c] Colonies and cultures grown in the absence of O_2.
[d] Fold reduction compared to wild type cultures grown in the absence of O_2.

recognized, both because such excisional Sm^r transformants can occur in higher numbers than the spontaneous revertants and because they become Kn^s (Table 2).

Spontaneous Sm^r clones in *rpsL* heterozygotes. The *kan-rpsL*$^+$ cassette was not completely stable in the heterozygous state. This was indicated by the composition of clones arising by introduction of the cassette into an Sm^r strain by transformation: cultures grown from single Kn^r colonies had a variable minority of Kn^r Sm^r cells (0.1 to 10 per 10,000). On subculturing single cells, all Sm^r subclones tested were Kn^r, while other Kn^r subclones were Sm^s, but again contained a variable but nonzero minority of Sm^r (data not shown). As Kn^r Sm^r arose as well in *comA* (noncompetent) cultures, the generation of the Sm^r minority did not depend on transformation but apparently arose by intracellular recombination events between the Sm^s and Sm^r *rpsL* alleles to convert Sm^s to Sm^r ("genuine → cassette" gene conversion) while leaving *kan* in place. Since such spontaneous Sm^r subclones would represent false-positives during the practical applications envisaged for this cassette, it is important to understand their source. It would be especially valuable to know how to minimize their occurrence when applying the method to mutate virulent isolates, in which the level of competence for genetic transformation may not be as high as in standard laboratory stocks.

Spontaneous Sm^r clones result from gene conversion. A frequency of 2.4 Sm^r clones per 10,000 Sm^s cells was observed through plating of several independent cultures of strain R960 (Table 1). Chromosomal DNA from Kn^r derivatives of strain R800 obtained through transformation with R974 DNA (Table 3) readily transformed strain R800 to Sm^r (data not shown), demonstrating linkage of Sm^r to Kn^r and strongly suggesting that the *rpsL41* allele had replaced the cassette-linked *rpsL*$^+$ copy. Consistent with this interpretation, introduction of the *kan*-linked *rpsL41* gene of R974 into strain R416 did not abolish its resistance to Sm (Table 3). R974 is therefore a candidate prototype for genuine → cassette spontaneous conversion of *rpsL* sequences. Other explanations, such as spontaneous mutation to Sm^r of the *kan*-linked *rpsL* copy, appeared unlikely inasmuch as the rate of mutation to Sm^r is very low in a wild-type background (13). In line with this interpretation, no spontaneous Sm^r could be obtained in strain R961, which harbors two *rpsL*$^+$ alleles, indicating that the presence of a resident *rpsL41* mutant allele was required for the production of Sm^r clones.

To characterize the conversions more precisely, we sequenced the cassette *rpsL* genes in spontaneous Sm^r revertants arising in cultures of CP1296, which are described above. As reported previously (13), the *rpsL1* allele of *rpsL* differs from the R6 wild-type gene sequence (Z15120) at three positions, although two of those are synonymous substitutions. Sequencing of the cassette copy of *rpsL* from four Sm^r revertants obtained from CP1296 revealed in each case that all three nucleotide substitutions characteristic of the distinctive *rpsL1* allele were present in the *kan-rpsL* cassette. Thus, the cassette *rpsL*$^+$ had been replaced by a copy of the genuine Sm^r *rpsL1* allele precisely, establishing the origin of spontaneous Sm^r as gene conversion, not mutation.

Sm^r spontaneous convertants were also observed in cultures of Kn^r derivatives of strain R800 obtained through transformation with R974 DNA (data not shown), indicating that the *rpsL41* allele linked to *kan* could be donated to the *rpsL*$^+$ gene. This observation suggested that cassette → genuine conversion could occur as readily as genuine → cassette conversion. Such a conversion would account for the recovery of CP1326, a spontaneous derivative of CP1296 that was stably Sm^s (Table 1). Indeed, sequencing both copies of *rpsL* in CP1326 revealed a wild-type allele at both sites, showing that all three divergent bases of the *rpsL1* allele had been replaced by R800 (*rpsL*$^+$) sequence, and confirmed the apparent conversion of the *rpsL1* gene to the sequence of the Sm^s allele carried in the *kan-rpsL*$^+$ cassette.

Spontaneous conversion is RecA dependent. To establish whether spontaneous conversion events occurred through homologous recombination, a *recA* derivative of the *rpsL*$^+$/*rpsL41* heterozygous strain R960 was constructed (strain R989; Table 1). To improve growth of the *recA* derivative, colonies and liquid cultures were grown in the absence of oxygen. The frequency of appearance of spontaneous Sm^r revertants was diminished 39-fold in this strain compared to the isogenic *recA*$^+$ parent similarly grown under anaerobic conditions (Table 4). Interestingly, the spontaneous conversion rate of the wild-type parent itself was diminished fivefold compared to the rate under aerobic conditions (Table 4). This observation suggests that oxygen could induce DNA lesions, possibly leading

TABLE 5. Mismatch repair-dependent reduction of conversion frequency using the *rpsL1-rpsL*$^+$ allele combination and a *hex*$^+$ genetic background

Strain	Genotype	Hex	Mismatch[a]	Sm^r frequency per 10,000 cells[b]	Fold reduction[c]
R960	*rpsL41 cbp3*::*kan-rpsL*$^+$ *hex*$^+$	+	HE	2.49 ± 0.62	1.0
R990[d]	*rpsL1 cbp3*::*kan-rpsL*$^+$ *hex*$^+$	+	LE	0.14 ± 0.020	18
		+	LE	0.11 ± 0.027	23
R1004	R960 but *hexA*::*cat*	−	HE	2.40 ± 0.63	1.0
R1005	R990 but *hexA*::*cat*	−	LE	1.95 ± 0.93	1.3

[a] HE, essentially not recognized or corrected by Hex; LE, transition mismatch recognized or corrected out by Hex.
[b] Calculated from four or five independent cultures grown from individual colonies resuspended in 2 ml of broth and grown to an OD_{550} of 0.5 to 0.7 before plating.
[c] Compared to the *rpsL41-rpsL*$^+$ combination (*hex*$^+$ background).
[d] Duplicate experiments on two different occasions are listed for R990.

to chromosome breaks and subsequent conversion events during repair.

To understand the residual source of Sm^r clones in the *recA* background, a representative spontaneous Sm^r derivative (R1001) of the *recA* mutant strain was retained for analysis. Transformation of the *kan-rpsL* cassette of R1001 into strain R416 did not abolish its resistance to Sm (Table 3), showing the lack of the *rpsL*$^+$ allele in the cassette. However, the cassette also did not carry an Sm^r gene, as no transformation of strain R800 to Sm^r could be obtained with chromosomal DNA from a Kn^r derivative of strain R800 generated through transformation with R1001 DNA (Table 3). Together, these results indicate that the *kan*-linked *rpsL* copy in strain R1001 was inactivated by mutation. We conclude that it is likely that all Sm^r revertants produced in a recombination-deficient *recA* background occur by this mechanism.

Spontaneous Sm^r can also arise in the wild-type background by inactivation of *rpsL*. One exceptional Sm^r revertant of CP1296, clone CP1296A4, did not contain *rpsL1* in the *kan-rpsL* cassette. Instead, sequencing of the cassette copy of *rpsL* in this exceptional revertant showed that it carried a single-base deletion at nucleotide (nt) 99 in *rpsL*, changing the sequence $T(A)_6GTT$ to $T(A)_5GTT$. The frameshift would be expected to inactivate the dominant *rpsL*$^+$ allele, explaining the loss of the dominant Sm^s phenotype in this revertant by gene inactivation.

Strategies to reduce spontaneous conversion frequency. Inasmuch as spontaneous conversion is RecA dependent (see above), it is likely to involve the formation of a transient heteroduplex structure between the two *rpsL* copies. We reasoned that inclusion of a mismatch normally recognized by the Hex mismatch repair system (2) within the heteroduplex structure leading to conversion would provoke Hex-dependent abortion of the recombination intermediate and therefore would reduce spontaneous conversion. This hypothesis was tested by comparing Sm^r frequencies in cultures of strain R960 (*kan-rpsL*$^+$/*rpsL41*, Table 1) to those in cultures of strain R990 (*kan-rpsL*$^+$/*rpsL1*, Table 1).

The *rpsL1* allele (transferred from strain CP1200 to the R800 background) contains two silent transitions in addition to

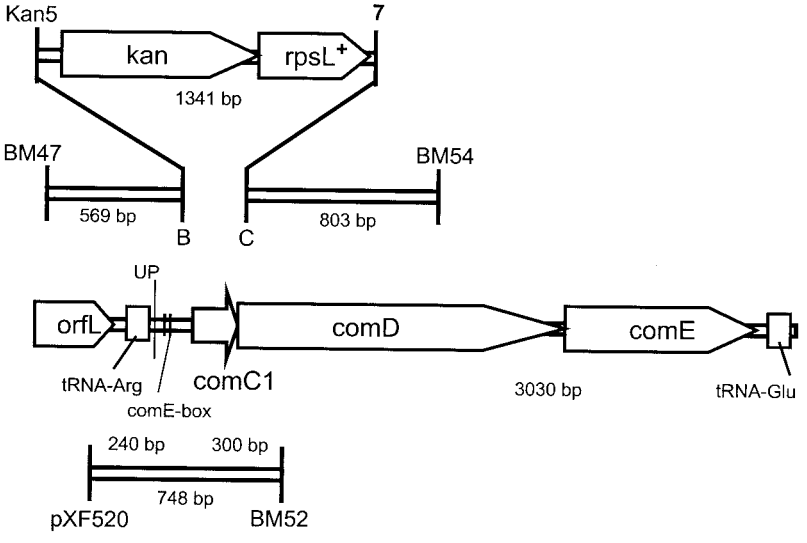

FIG. 2. Use of Janus at the *comCDE* chromosomal locus. Top, locations of primers used to generate a 2,669-bp-long PCR product (see Materials and Methods) for the construction of strain R1029. R1029 harbors a substitution of the *comC* gene by the *kan-rpsL*$^+$ cassette. Middle, map of the *comCDE* chromosomal region (16) showing limits of PCR fragments used and the site of the UP mutation previously characterized as a single nucleotide change within the terminator of the $tRNA^{Arg}$ located upstream of *comC* (12). Bottom, limits of the homologous segment carried by the cat3-BM52 PCR fragment and used to transfer the UP mutation into strain R1029. pXF520 refers to the limit of the pneumococcal insert in the nonreplicative plasmid pXF520 (16), which is carried in strain R810 as an insertion in *comC*.

TABLE 6. Transfer of a silent point mutation at the *comCDE* locus: use of Janus-containing strain R1036

Donor DNA	Growth (CFU/ml) with selection on:			Sm^r frequency per 10,000 cells	Fold increase in Sm^r frequency
	None	Sm	Rif[a]		
R304 chromosomal DNA	1.56×10^8	2.44×10^6	5.8×10^5	156	246.5
cat3-BM52 (UP) PCR fragment		2.02×10^4		1.3	2.04[b]
None		9.9×10^3		0.63[c]	1

[a] The ratio Rif^r/Sm^r is normally close to 0.15 in a *hex*$^+$ recipient because Hex-dependent mismatch repair affects integration of the *rif23* marker more severely than of *rpsL41*. The value (0.24) obtained here was expected, as Sm^r transformants do not result from substitution of the genuine copy of *rpsL* by the *rpsL41* allele but from excisional replacement of the *kan-rpsL* cassette by *comC*$^+$.

[b] Four Sm^r clones were further analyzed by PCR. Three corresponded to substitution of the *kan-rpsL*$^+$ cassette by the *comC*$^+$ segment (excision events) and harbored the UP mutation (see Fig. 2); the fourth clone resulted from a spontaneous conversion event and had retained the *kan-rpsL* cassette.

[c] A lower spontaneous conversion frequency (0.12 per 10,000 cells) was measured in four independent cultures of strain R1036. This value is totally consistent with spontaneous conversion frequencies measured at the *cbp3* locus using the *rpsL1-rpsL*$^+$ mismatched combination and a *hex*$^+$ recipient (see Table 5).

the same single base change as that causing Sm^r in *rpsL41* (13). While the *rpsL41*/*rpsL*$^+$ mismatch is not efficiently recognized by Hex, transition mismatches are known to be corrected at the heteroduplex stage in transformation (2). The *kan-rpsL*$^+$/*rpsL1* combination was found to reduce the frequency of conversion to Sm^r 18-fold (Table 5), and this reduction depended on the Hex phenotype of the strain (compare strains R990 and R1005, Table 5). This result further supports the interpretation that spontaneous Sm^r strains arise by gene conversion and shows directly that the combined use of a *hex*$^+$ recipient together with an *rpsL*$^+$/*rpsL1* mismatched heterozygous configuration significantly reduces the spontaneous conversion frequencies and can help to lower the background of Sm^r clones if and when necessary.

Interestingly, the Sm^r revertants may be enriched during colonial culture growth; pure Sm^s populations of CP1296 grown in liquid culture exhibited a lower frequency of revertants (7.5×10^{-6}) than parallel colonial cultures (1.5×10^{-4}) (data not shown), offering a possible alternative to the Hex-dependent strategy for reducing the rate of gene conversion.

Use of Janus to place a regulatory mutation at the *comCDE* chromosomal locus. To demonstrate use of Janus for the transfer of a silent mutation not conferring a directly selectable phenotype, an insertion of the cassette was prepared at the *comC* (Δ*comC*::*kan-rpsL*$^+$; see Materials and Methods) gene to form strain R1036. The three genes, *comC*, *comD*, and *comE*, encode the competence-regulating peptide signal (6), its receptor, and the cognate response regulator, respectively (16). For the excisional transformation step, a 748-bp region overlapping *comC* and carrying the UP mutation previously characterized as a single nucleotide change within the terminator of the tRNA^{Arg} located upstream of *comC* (12) was amplified from the *comCDE* chromosomal region of strain R810 with the cat3-BM52 primer pair (Table 1). This fragment was used in transformation of strain R1036 (Δ*comC*::*kan-rpsL*$^+$ *rpsL1 hex*$^+$; Table 1) to replace the *kan-rpsL* cassette while reintroducing the *comC* gene at its normal chromosomal location together with the silent UP mutation (Fig. 2 and Table 6). As the lengths of homologous region allowing this exchange were not large (240 bp in the region upstream of *comC* and 300 bp

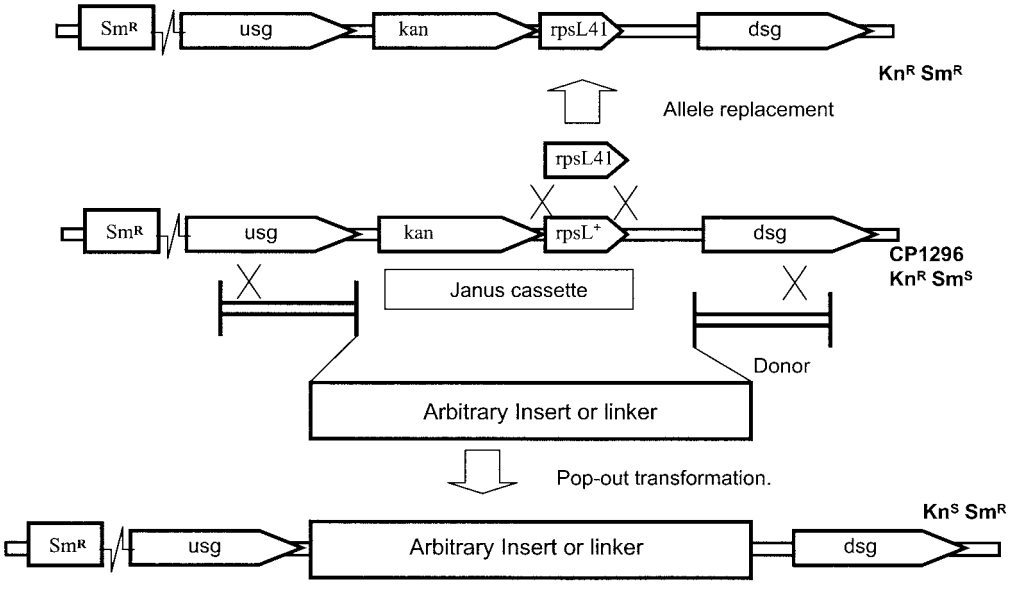

FIG. 3. Two fates of Janus. Possible recombination mechanisms for generation of Sm^r derivatives are illustrated, dependent on (top) gene conversion or (bottom) transformation by exogenous DNA. Crosses show limits of possible single-strand integration or gene conversion events.

downstream of it) (Fig. 2), the Hex strategy was used to reduce the background rate of gene conversion to a level comparable to the transformation frequency of this small donor fragment. With this strategy, transfer of the UP mutation was readily obtained (see Table 6 footnote *b*).

DISCUSSION

To construct mutant strains of *S. pneumoniae* without introducing new antibiotic resistance markers, the Janus cassette can be used in a two-step transformation procedure. The first transformation into an Sm^r strain using the cassette disrupts or tags a target gene on the chromosome by homologous recombination targeted by flanking DNA homology. This Janus strain can subsequently become Sm^r by two principal routes, as illustrated in Fig. 3. In a second transformation, deletion of the cassette by homologous recombination will restore Sm resistance, allowing construction of mutations without a selectable phenotype. Alternatively, gene conversion eliminating the dominant Sm^s allele can lead to spontaneous accumulation of Sm^r clones in a culture of a Janus heterozygote. This conversion is specific, depends on RecA, and involves an intermediate subject to Hex correction. Because Sm^r revertants accumulate during growth of $Sm^{r/s}$ heterozygotes, it may be useful to minimize their frequency in cultures of the intermediate Sm^s strain by choosing a low-efficiency allele of the Sm^r locus in a hex^+ strain or by controlled subculturing. Since Sm selection after the second transformation step is simply selecting for loss of the cassette, the design of the donor DNA used in that step is broadly unrestricted as long as it includes terminal segments of homology flanking the cassette. Deletions of additional sequences adjacent to the cassette are possible, for example, and virtually any gene(s) (or none) could be inserted in its place.

The *kan-rpsL* cassette described here may be useful in applications other than the "drop-in/pop-out" mutagenesis strategy for which we designed it. For example, it is interesting that Sm^s insertions create strains that could detect and "extract" the wild-type alleles of specific genes from any pneumococcal DNA source, with production of Sm^r transformants. Also, as illustrated by the experiments reported here with *hex* and *recA*, *rpsL* heterozygotes permit sensitive monitoring of recombination rates independent of the process of genetic transformation.

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