Contraselectable Streptomycin Susceptibility Determinant for Genetic Manipulation and Analysis of *Helicobacter pylori*

Daiva Dailidiene, Giedrius Dailide, Dangeruta Kersulyte, and Douglas E. Berg*

*Departments of Molecular Microbiology, Genetics, and Medicine, School of Medicine,
Washington University, St. Louis, Missouri 63110*

Many *Helicobacter pylori* genetic studies would benefit from an ability to move DNA sequences easily between strains by transformation and homologous recombination, without needing to leave a conventional drug resistance determinant at the targeted locus. Presented here is a two-gene cassette that can be selected both (i) against, due to a *Campylobacter jejuni rpsL* gene (dominant streptomycin susceptibility in cells also carrying an *rpsL-str*[r] allele), and (ii) for, due to an *erm* gene (erythromycin resistance). This *rpsL,erm* cassette's utility was assessed by using it to replace four gene loci (*mdaB*, *frxA*, *fur*, and *nikR*) in four streptomycin-resistant [Str[r]] strain backgrounds (derivatives of 26695, SS1, X47, and G27MA). The resultant 16 strains (phenotypically erythromycin resistant [Erm[r]] and Str[s]) were each transformed with wild-type genomic DNAs, and Str[r] derivatives were selected. The desired Erm[s] Str[r] isolates were obtained at frequencies that ranged from 17 to 96% among Str[r] transformants, with the Erm[s] yield apparently depending on the strain background and genome location of the targeted locus. The ease of isolating unmarked transformants described here should be valuable for many *H. pylori* molecular genetic and evolutionary analyses.

Genetic studies of the gastric pathogen *Helicobacter pylori* (21) often entail (i) the construction of cloned DNAs or PCR products containing a drug resistance determinant inserted into or near a gene of interest, (ii) DNA transformation, and (iii) selection for resistant transformants, which arise by homologous recombination and replacement of recipient DNA sequences by corresponding sequences from donor DNA. Although this strategy has been used in hundreds of *H. pylori* studies, successfully overcoming the rarity of natural transformation events, it becomes seriously limiting or flawed in at least four interesting situations, in each case because transformants retain resistance determinants at targeted loci: (i) if changes at numerous loci in the same strain are needed, e.g., in studies of phenotypes determined by multiple genes with additive or redundant effects (because only a few selectable resistance markers are available for *H. pylori*); (ii) if donor resistance determinants might affect downstream gene expression, or cellular physiology more generally (e.g., in many cases the resistance enzymes use cellular metabolites to modify antibiotics and thereby confer resistance to them [9]); (iii) if alleles with subtle (e.g., point mutation) differences are to be compared; or (iv) if alleles from many strains are to be studied in a common genetic background. Such limitations can be overcome by using a recipient strain that contains a contraselectable marker at the locus of interest. This allows the desired transformants to be selected by the loss of this recipient marker, thereby bypassing the need for a conventional resistance determinant in donor DNA.

Three genes that have been used for contraselection in other systems are *thyA* (thymidylate requirement; trimethoprim sensitivity) (4, 34), *sacB* (sucrose sensitivity) (5, 27, 33), and *rpsL* (streptomycin sensitivity) (25, 33). *H. pylori* is naturally trimethoprim resistant. This could be ascribed to (i) its apparent lack of a *thyA* gene (based on BLASTP homolog searches [31]); (ii) an intrinsic resistance of its enzyme for reduced folate synthesis, an apparent dihydrofolate reductase-dihydropteroate synthase chimaera (18); and/or (iii) other factors. An early report had indicated that *sacB* could serve as a contraselectable marker in *H. pylori* but did not describe the details of efficiency or complications that may have been encountered (8). In our *H. pylori sacB* experiments, however, selection for resistance to a range of sucrose concentrations gave far more background growth of nominally sensitive cells than was expected based on *Escherichia coli* experiences (33, 34), and conditions for sucrose-based differential killing of *sacB*-containing *H. pylori* cells varied among trials (unpublished data). Other groups had also found contraselection of *sacB* to be difficult in *H. pylori* although better than having no such marker at all (T. L. Cover, unpublished data; R. Haas, unpublished data). A third contraselection strategy, implemented here, uses the *rpsL* (ribosomal protein S12) gene and is based on the dominance of wild-type streptomycin-sensitive (Str[s]) alleles to resistance-conferring mutant alleles (16, 25).

Historically, streptomycin killing has been associated with diverse effects, including membrane damage and irreversible streptomycin uptake. Paradoxically, these were all blocked by the bacteriostatic translation inhibitor chloramphenicol. This implicated the capacity to synthesize proteins, even though streptomycin also inhibited protein synthesis. Sublethal streptomycin concentrations suppressed nonsense mutations, increased mutation, and caused membrane fragility. Biochemical studies showed that streptomycin allowed the continuation of translation once begun, but with errors in translation (misreading), and that it also allowed ribosomes to bind to mRNAs but blocked them from initiating translation (10). Davis' early uni-

* Corresponding author. Mailing address: Department of Molecular Microbiology, Campus Box 8230, Washington University Medical School, 4940 Parkview Place, St. Louis, MO 63110. Phone: (314) 362-2772. Fax: (314) 362-1232. E-mail: berg@borcim.wustl.edu.

fying explanation for streptomycin's lethality (10) focused on errors it induced during translation and a resultant accumulation of defective proteins. He focused in particular on membrane proteins, interpreting that defects in them were responsible for the disruption of cell membrane integrity, massive leakage of ions and molecules, and cell death. This could explain the dominance of streptomycin sensitivity over resistance, as could the binding of Str^s ribosomes to mRNAs, blocking the access of resistant ribosomes to these mRNAs, failure to translate them, and their ensuing degradation (10).

Recent exquisitely detailed molecular and genetic analyses provide mechanistic understanding. Streptomycin binds several specific 16S rRNA loops and thereby diminishes ribosome flexibility and the changes in conformation that charged tRNA binding normally induces. It stabilizes a "ribosomal ambiguity" (*ram*) state in which there is little if any of the proofreading needed for error-free protein synthesis (7, 15, 22, 24). Protein S12, which is mutated in Str^r strains, binds rRNA sequences near sites of streptomycin binding. Most positions in which change confers resistance are in S12 domains that interact with the rRNA target. In addition, most Str^r mutant S12 proteins increase the accuracy of translation, apparently by destabilizing the *ram* state that streptomycin itself induces; the most extreme of such *rpsL* (S12) alleles make growth streptomycin dependent. This can be overcome by mutations in ribosomal proteins S4 and S5, that in turn stabilize the *ram* state in this dynamic and finely tuned ribonucleoprotein machine (7, 14).

The two S12 residues that are most frequently changed in streptomycin-resistant *H. pylori* are Lys43 (Lys42 in *E. coli* numbering), which contacts the rRNA-bound streptomycin directly, and Lys88 (Lys87 in *E. coli* numbering), which is nearby in the structure (7, 14, 32). Although the Lys-to-Arg changes at these codons seem to be quite innocuous (14), other amino acid replacements can markedly diminish bacterial fitness. In particular, a large fitness cost and a resulting selection for compensatory mutations in other genes have been documented in *E. coli* and *Salmonella enterica* serovar Typhimurium using strains with Lys42 replaced with Asn (19, 26) (not Arg). More critically, the Lys88Arg replacement used here, and also the Lys43Arg allele, have each been incorporated into *H. pylori* strains used for mouse infections, without obvious negative effects on fitness (12, 13, 23).

Streptomycin contraselection for chromosomal gene replacement had been developed for *H. pylori* previously (13) but has not been much used, perhaps because very few of the Str^r isolates recovered after transformation had sustained the desired replacement. This, we suspected, was due to the near identity of the *str^s* and *str^r* *H. pylori* *rpsL* alleles used: frequent gene conversion between them would result in unwanted Str^r gene convertants vastly outnumbering the desired Str^r transformants. If correct, it seemed that the *rpsL* gene might still be useful for *H. pylori* genetic engineering if the *rpsL* gene conversion could be reduced.

Generalized recombination (gene conversion included) depends on close matches between participating DNA molecules and is reduced by sequence divergence, even in species that, like *H. pylori*, lack the ability to cleave mismatch-containing (heteroduplex) DNAs (11, 20, 31). The *rpsL* genes of *H. pylori* and *Campylobacter jejuni* differ by some 18% in overall DNA sequence, but the encoded S12 proteins are 95% similar in
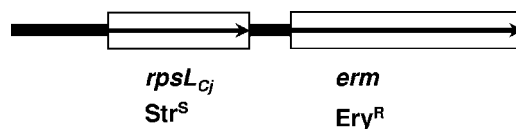

FIG. 1. Structure of the 1.5-kb *rpsL,erm* cassette, which confers dominant streptomycin susceptibility and selectable erythromycin resistance. Open boxes designate open reading frames; solid line indicates noncoding sequences. The cassette contains 272 bp of *C. jejuni* sequence upstream of the *C. jejuni rpsL* gene (408 bp), a 145-bp spacer, and then the 735-nucleotide *erm* gene.

amino acid sequence. The present study was initiated with an expectation or hope that *H. pylori* ribosomes containing the *C. jejuni* S12 protein would be functional and Str^s and that DNA sequence divergence between the *rpsL* genes of *C. jejuni* and *H. pylori* would diminish gene conversion sufficiently for the effective recovery of transformants by streptomycin contraselection. The *rpsL,erm* (streptomycin susceptibility, erythromycin resistance) cassette that we constructed and tested in these studies is diagrammed in Fig. 1.

MATERIALS AND METHODS

***H. pylori* strains and general methods.** The *H. pylori* strains used here were cultured at 37C in a standard microaerobic (5% O_2, 10% CO_2) atmosphere on brain heart infusion–horse blood agar plates, with 0.4% Isovitalex and the antibiotics amphotericin B (8 μg/ml), trimethoprim (5 μg/ml), and vancomycin (6 μg/ml). Erythromycin (10 μg/ml) and streptomycin (10 μg/ml) were added to this agar as needed to select for transformants or screen colonies for resistance to either drug. Natural transformation was carried out by adding ~1 μg of genomic DNA or PCR product to a lawn of cells growing exponentially on nonselective medium and, after overnight incubation, restreaking the population on selective (streptomycin- or erythromycin-containing) medium to obtain transformant colonies. Standard methods were used for PCR and for sequencing of the PCR products (2, 30).

Four unrelated *H. pylori* strains were used here, each chosen because it is much used in other molecular genetic studies: the genome-sequenced reference strain 26695 (31), which we use to identify the numerous genes that contribute to high-level metronidazole resistance (2); unrelated strains SS1 and X47 (X47 was originally called X47-2AL) (12, 17), which colonize mice, preferentially occupy different regions of the stomach (1, 28), and differ in the phenotypic consequences of inactivation of at least certain metabolic genes (30); and G27MA, a cell-culture-adapted derivative of the G27 strain that is frequently used for studies of *H. pylori*-host cell interaction (3) and that can efficiently colonize DBA/2 mice (W.-K. Lee, D. Dailidiene, and D. E. Berg, unpublished data). The *erm* gene used in the *rpsL,erm* cassette (Fig. 1) was originally from plasmid pRH151, kindly provided by Rainer Haas, and was matched in sequence to that of pNE131 (GenBank accession no. NC_001390). The *C. jejuni rpsL* gene used in this cassette was from plasmid pDRF265, kindly provided by David Hendrixson and Victor DiRita.

To prepare for the development and testing of the *rpsL,erm* cassette, an *str^r* allele of the normal chromosomal *H. pylori rpsL* gene was constructed and introduced into strain 26695 to generate 26695-str. This was achieved by PCR of *rpsL* DNA from 26695 with the primers rpsL1 and rpsL2 (Table 1) to generate a 203-bp PCR product that contained the A-to-G changes at each of the two sites most frequently responsible for streptomycin resistance (codons 43 and 88; Lys to Arg in each case) (32). This product was used to transform strain 26695 to streptomycin resistance to generate "26695-str." PCR-based DNA sequencing showed that each of several such 26695-str transformants contained an A-to-G change at nucleotide position 263 but was wild type at nucleotide position 129 (i.e., a change of Lys to Arg at codon 88 only). Genomic DNA from one such transformant was used to transform SS1 and G27MA to streptomycin resistance (generating SS1-str and G27MA-str). Strain X47 is already streptomycin resistant (10) and was found to contain the same *rpsL* A263G (Lys88Arg) allele.

PCR-based construction of strains with insertion and deletion alleles. The alleles used here were constructed by assembling individual PCR products without the need for recombinant DNA plasmid cloning, as first described by Chalker et al. (6). In brief, assembly depends on overlaps of ≥20 bp at the ends of DNAs

TABLE 1. PCR primers used in this study and features of their amplification products

Process	Primer[a]	Sequence (5′-3′)[b]	Product size and/or comments
Creation of streptomycin resistance allele (str[r]) of *H. pylori* rpsL gene	rpsL1	AGGGGTTTGTACTAGGGTTTATACGACTAC CCCTAA**G**AAGCCTAACTCG-3′	203 bp; the *rpsL* fragment containing the Lys43Arg mutation (AAA129AGA) str[r] allele in primer rpsL1 and the Lys88Arg mutation (AAG263AGG) str[r] allele in rpsL2 are shown (mutations are in boldface)
	rpsL2	GAACGATGTGGTATTTCACACCGGGTAAAT CCCTAACC**T**ACCCCCACG-3′	
H. pylori rpsL gene sequencing	rpsL-F	GGGAACAGGCATGTATAAGA-3′	675 bp
	rpsL-R	CGTCGAACATCATCTTATTGAT-3′	
erm gene amplification from plasmid pRH151	erm-F	CAATAATCGCATCAGATTGCAGTA-3′	853 bp; the 5′ ends of the erm-F and erm-R primers are 118 bp upstream of the 5′ end and exactly at the 3′ end of the *erm* open reading frame
	erm-R	TTACTTATTAAATAATTTATAGCTATTGAA-3′	
Δ*mdaB-erm* allele construction	1. m1	CCTTCTACCATTAAAATGTAATTG-3′	454 bp
	2. mermF1	*TACTGCAATCTGATGCGATTATTG***CTAATTAAG GAGTGGTCATGTTC**-3′	
	3. erm-F	CAATAATCGCATCAGATTGCAGTA-3′	853 bp
	4. erm-R	TTACTTATTAAATAATTTATAGCTATTGAA-3′	
	5. mermR1	*TTCAATAGCTATAAATTATTTAATAAGTAA***GGCTTGTTTATTCCACAATAAAGTC**-3′	526 bp
	6. m4	GAGCTTATGGAAGAATACAGCTCCTTG-3′	
	All		The 5′ ends of primers are as follows: primer 1, 596 bp upstream of the 5′ end of *mdaB*; primer 2, 165 bp upstream of the 5′ end of *mdaB*; primer 5, 62 bp downstream of the 3′ end of *mdaB*; primer 6, 563 bp downstream of the 3′ end of *mdaB*; the PCR products with primers 1 and 6 are 1,744 and 1,833 bp long from the wild-type and Δ*mdaB-erm* DNAs, respectively
Δ*mdaB-rpsL,erm* allele construction (*C. jejuni* rpsL insertion in the Δ*mdaB-erm* allele)	1. m1	CCTTCTACCATTAAAATGTAATTG-3′	454 bp
	2. mrpsL	CTAATTAAGGAGTGGTCATGTTC-3′	
	3. rpsL$_{CJ}$-F	*GAACATGACCACTCCTTAATTAG***GATGCTTTAT AACTATGGATTAAACAC**-3′	686 bp
	4. rpsL$_{CJ}$-R	*TACTGCAATCTGATGCGATTATTG***ATCTAACGG ATTTGTCTGTATG**-3′	
	5. erm-F	CAATAATCGCATCAGATTGCAGTA-3′	1,379 bp
	6. m4	GAGCTTATGGAAGAATACAGCTCCTTG-3′	
	All		The PCR products with primers 1 and 6 are 1,744 and 2,518 bp long from the wild-type and Δ*mdaB-rpsL,erm* DNAs, respectively; primers 3 and 4 were used for amplification of the *C. jejuni* rpsL gene from plasmid DRH265; the 5′ ends of primers 3 and 4 are 273 bp upstream of the 5′ end and 27 bp downstream of the 3′ end of the *rpsL* open reading frame
Δ*nikR-rpsL,erm* allele construction (replacement of *nikR* with the rpsL,erm cassette)	1. 1338A1-F	TAATAAGCCCACATAAGGCGCG-3′	547 bp
	2. 1338A2-R	*TGTTTAATCCATAGTTATAAAGCATC***ATCCTTT TTTGGCATGAGTTCG**-3′	
	3. rpsL-F-1	GATGCTTTATAACTATGGATTAAACAC-3′	1,539 bp
	4. erm-R	TTACTTATTAAATAATTTATAGCTATTGAA-3′	
	5. 1338A3-F	*AATAGCTATAAATTATTTAATAAGTAA***GGGGTT AAATTCGCTAAATTGAC**-3′	481 bp
	6. 1338AA4-R	TTGGATCTCTTCATAGCCAATCC-3′	
	All		The 5′ ends of the primers are as follows: primer 1, 556 bp upstream of the 5′ end of *nikR*; primer 2, 10 bp upstream of the 5′ end of *nikR*; primer 5, inside *nikR*, 54 bp from the 3′ end; primer 6, 427 bp downstream of the 3′ end of *nikR*; the PCR products with primers 1 and 6 are 1,430 and 2,567 bp long from the wild-type and Δ*nikR-rpsL,erm* DNAs, respectively
Δ*frxA-rpsL,erm* allele construction (replacement of *frxA* with the rpsL,erm cassette)	1. frxA1-F	GTGCGCTTCAAAGCTTGGGTTA-3′	444 bp
	2. frxA2-R	*GTGTTTAATCCATAGTTATAAAGCATC***GCAACC ACTTGTTCTCTGTCCA**-3′	
	3. rpsL-F-1	GATGCTTTATAACTATGGATTAAACAC-3′	1,539 bp
	4. erm-R	TTACTTATTAAATAATTTATAGCTATTGAA-3′	
	5. frxA3-F	*TCAATAGCTATAAATTATTTAATAAG*TAA**GCTT GGCGTTAGCCAAGTGCT**-3′	275 bp
	6. frxA4-R2	GCCTTCAATGTTGCGCTCTTTGT-3′	
	All		The 5′ ends of the primers are as follows: primer 1, 421 bp upstream of the 5′ end of *frxA*; primer 2, within *frxA*, 23 bp from its 5′ end; primer 5, 36 bp downstream of the 3′ end of *frxA*; primer 6, 311 bp downstream of the 3′ end of *frxA*; the PCR products with primers 1 and 6 are 1,386 and 2,258 bp long from the wild-type and Δ*frxA-rpsL,erm* DNAs, respectively

TABLE 1—*Continued*

Process	Primer[a]	Sequence (5′-3′)[b]	Product size and/or comments
Δ*fur-rpsL,erm* allele construction (replacement of *fur* with *rpsL,erm* cassette)	1. x5k-F	CCTTAATTTAGCCGCTTCTTGTTTG-3′	553 bp
	2. fur-R-A2	*AAGTGTTTAATCCATAGTTATAAAGCATC* **CTGATATCTTCCTTATCCGTA**-3′	
	3. rpsL-F-1	GATGCTTTATAACTATGGATTAAACAC-3′	1,539 bp
	4. erm-R	TTACTTATTAAATAATTTATAGCTATTGAA-3′	
	5. fur F-A3	*TTCAATAGCTATAAATTATTTAATAAGTAA***GCT** **TAGATAGGGCTATCTTT**-3′	454 bp
	6. x4-R	CTGTAGAGTTGCCTGGAATTTATCA-3′	
	All		The 5′ ends of the primers are as follows: primer 1, 554 bp upstream of the *fur* 5′ end; primer 2, 2 bp upstream of the *fur* 5′ end; primer 5, 18 bp downstream of the *fur* 3′ end; primer 6, 471 bp downstream of the *fur* 3′ end; the PCR products with primers 1 and 6 are 1,478 and 2,546 bp long from the wild-type and Δ*frxA-rpsL,erm* DNAs, respectively

[a] Generic primer designations 1, 2, 3, 4, 5, and 6 for the construction of insertion and deletion alleles are as diagrammed in Fig 2.

[b] The portions of the primer sequences in italics constitute complement with another primer (primer 2 with primer 3 or vice versa and primer 4 with primer 5 or vice versa); the portions of these hybrid primers in boldface indicate regions matching the *H. pylori* genomic DNA used for amplification. All, primers 1 to 6.

to be joined together, which, in turn, result from the design of PCR primers used in amplification (see Table 1, sequences in italics) (6, 29).

To construct the *rpsL,erm* cassette, we first replaced the *mdaB* quinone reductase gene in the *H. pylori* chromosome with the *erm* gene and then used genomic DNA carrying this Δ*mdaB-erm* allele for PCR to insert the *C. jejuni rpsL* gene just upstream of *erm*. Each of these manipulations involved a three-fragment assembly as in Fig. 2, transformation and selection for erythromycin resistance (Erm^r), and PCR verification of structure. Two sequential three fragment assemblies were used because three-fragment assemblies have been more reliable for us than four-fragment assemblies.

In detail, for the first stage and using the nomenclature presented in Fig. 2) PCR products were generated with primers 1 and 2 (fragment A, upstream of *mdaB*), primers 5 and 6 (fragment C, downstream of *mdaB*), and primers 3 and 4 (fragment B, *erm*) (Fig. 2; the primers are listed in Table 1). Primers 2 and 3 overlap, as do primers 4 and 5. A second round of PCR amplification using a mixture of fragments A, B, and C and primers 1 and 6 only yielded a 1.8-kp product. Transformation of strain 26695 with this product and selection for erythromycin resistance resulted in replacement of *mdaB* with the Δ*mdaB-erm* allele.

The *C. jejuni rpsL* gene was inserted upstream of *erm* in the Δ*mdaB-erm* allele to generate the 1.5-kb *rpsL,erm* cassette (Fig. 1). This was achieved by amplification of three PCR products and their assembly into a composite PCR product (again as in Fig. 2 and Table 1), use of this product to transform strain 26695-str, selection of Erm^r transformant colonies, and identification of those products that had become phenotypically Str^s. In terms of the depiction in Fig. 2, fragment A contained sequences just upstream of *erm* in the Δ*mdaB-erm* allele; fragment C contained *erm* and also *H. pylori* sequences downstream of *mdaB*; and fragment B contained the *Cj.rpsL* (*str^s* allele) gene. PCR of the mixture of segments A, B, and C with primers 1 and 6 yielded the desired 2.5-kb assembly.

Replacements of genes *frxA*, *fur*, and *nikR* with the *rpsL,erm* cassette were made similarly by (i) using DNAs from upstream and downstream of these target genes (amplified with appropriate primers 1 and 2 and primers 5 and 6) and also from the *rpsL,erm* cassette (amplified with primers 3 and 4); (ii) assembly from the three-fragment mixture using the appropriate locus-specific primers 1 and 6 (Table 1); (iii) transformation of 26695-str, selection of Erm^r colonies, and identification of those that were phenotypically Str^s; and (iv) verification by PCR of the replacement of wild-type alleles by the *rpsL,erm*-marked deletion alleles.

RESULTS

A two-gene (1.5-kb) cassette containing the *C. jejuni rpsL* gene (*str^s* allele) upstream of an erythromycin resistance gene (*erm*) (Fig. 1), flanked by *H. pylori* chromosomal sequences that normally flank the *mdaB* quinone reductase gene (allele designated Δ*mdaB-rpsL,erm*) was constructed by PCR (Fig. 2) and recovered in the *H. pylori* chromosome after the transformation of 26695-str (see Materials and Methods). PCR tests showed that each of four Erm^r Str^s transformants tested contained the expected Δ*mdaB-rpsL,erm* allele in place of the full-length *mdaB* gene. Streaks of such colonies to streptomycin-containing agar typically yielded a few Str^r derivative colonies after prolonged incubation. This slight instability is not seen in haploid strains containing only an *rpsL-str^s* allele. These rare Str^r derivatives remained Erm^r and, based on PCR tests, retained the Δ*mdaB-rpsL,erm* allele in place of *mdaB*. These derivatives likely result from occasional gene conversion, despite *H. pylori*-*C. jejuni rpsL* sequence divergence.

In initial characterizations, nine recipient populations, each representing a different Str^s Δ*mdaB-rpsL,erm* transformant of 26695-str, were used for transformation with genomic DNAs from wild-type 26695. About 30 Str^r colonies from each transformation were streaked with toothpicks to erythromycin-containing agar: between 63 and 96% of Str^r colonies obtained
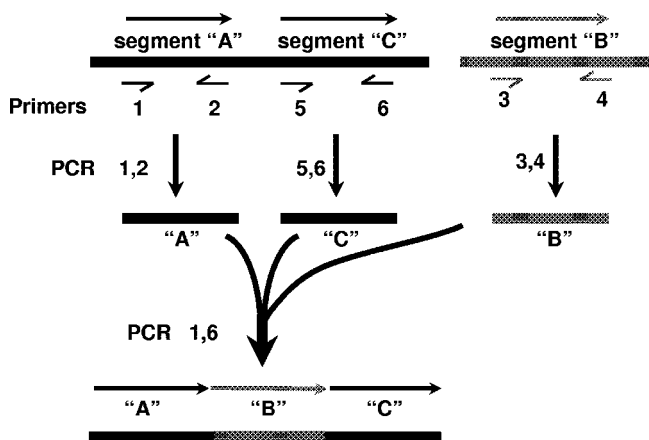

FIG. 2. Construction of insertion and deletion alleles by assembly of three fragments with overlapping ends. Half arrows indicate the positions of the primers, whose sequences are given in Table 1. Segments A and C represent DNA segments flanking the locus to be deleted or the site of insertion (depending on needs of experiment). Segment B represents (i) the *erm* (resistance) gene used initially to replace the *mdaB* locus; (ii) the *rpsL* streptomycin susceptibility gene from *C. jejuni* that was inserted just upstream of *erm* in a strain carrying *erm* in place of *mdaB*; or (iii) the *rpsL,erm* cassette, which can be moved to many loci.

TABLE 2. Yields of Erm^s isolates among Str^r colonies
after transformation[a]

$rpsL,erm$ marked gene deletion	Yield of Str^s Erm^r ($rpsL,erm$) derivative of recipient strain ± SD:			
	26695-str	SS1-str	X47	G27MA-str
$mdaB$	0.84 ± 0.13 (9)	0.40 ± 0.10 (2)	0.88 ± 0.14 (2)	0.21 ± 0.14 (4)
$frxA$	0.53 ± 0.22 (10)	0.90 ± 0.04 (2)	0.87 ± 0.03 (2)	0.17 ± 0.15 (4)
fur	0.28 ± 0.3 (8)	0.22 ± 0.11 (2)	0.71 ± 0.03 (2)	0.56 ± 0.26 (4)
$nikR$	0.54 ± 0.42 (2)	0.86 ± 0.18 (2)	0.96 ± 0.02 (2)	0.38 ± 0.35 (4)

[a] Average fraction of Str^r colonies ± standard deviation that were Erm^s after transformation of the indicated $rspL,erm$ cassette containing recipient strain with isogenic wild-type genomic DNA. These Erm^s colonies were products of transformation and replacement of the contraselectable cassette, not gene conversion. These fractions are based on erythromycin resistance tests of approximately 30 colonies per transformation experiment. Listed in parentheses are the numbers of independent transformations, each with a different single colony isolate of the indicated recipient strain.

were Erm^s (average, 84% ± 13%) (Table 2). PCR tests confirmed $\Delta mdaB$-$rpsL,erm$ replacement by full-length $mdaB$ in each of eight Str^r Erm^s colonies tested, and retention of $\Delta mdaB$-$rpsL,erm$ in four Str^r Erm^r colonies, as expected (Fig. 3). In related tests, PCR products containing full-length $mdaB$ plus several hundred base pairs of flanking sequences (made with $mdaB$ primers 1 and 6; Table 1) were used in equivalent transformations of $\Delta mdaB$-$rpsL,erm$ derivatives of 26695-str or isogenic metronidazole-resistant strains (mutation in $mdaB$ is implicated in the development of high-level metronidazole resistance [2]). Approximately 30 to 86% of the Str^r colonies obtained (average of 68%; ten transformations) were of the desired Erm^s type.

To test the generality of this allele replacement strategy, the $\Delta mdaB$-$rpsL,erm$ allele was moved by transformation into the streptomycin-resistant ($rpsL$) strains SS1-str, G27MA-str, and

X47 by using genomic DNA from 26695-str $\Delta mdaB$-$rpsL,erm$ and selection for erythromycin resistance. Each of the ~30 Erm^r transformants in each of these strains were Str^s, as expected. Two $\Delta mdaB$-$rpsL,erm$ derivatives of each strain, found by PCR to have the expected structure, were used as recipients in transformations with DNA from isogenic wild-type parents. Table 2 shows that the yields of Str^r Erm^s transformants among Str^r colonies ranged from ~21% in the case of G27MA-str to 88% in the case of X47.

To further test the general utility of this contraselection strategy, we constructed deletion alleles marked with the $rpsL,erm$ cassette of three other genes—$frxA$ (nitroreductase), fur (iron- and pH-responsive regulation), and $nikR$ (nickel-responsive regulation)—by PCR (Fig. 2) and transformation of 26695-str (Materials and Methods). DNAs from transformants with the desired Str^s phenotypes and PCR-verified structures were then used to move each $rpsL,erm$-tagged deletion allele to the three other strains (G27MA-str, SS1-str, and X47). The yields of Erm^r Str^s transformants among Erm^r colonies made with these genomic DNAs varied from ~50% to ~100% (data not shown). Since just one genomic DNA preparation for each locus was used to move a given allele among $H. pylori$ strains (in each case from the 26695-str derivative), this variation in Str^s colony yield might reflect an interplay of the strain background (e.g., nuclease or DNA repair activities) and the sequence of the transforming DNA and/or the targeted region.

These new $rpsL,erm$-marked strains were then transformed with isogenic wild-type genomic DNAs, and Str^r colonies were selected and tested as described above. Table 2 shows that the yields of Erm^s Str^r transformants among Str^r colonies varied with the locus under study (from ~17% to ~90%) and with the strain background. PCR tests of a few representative Str^r Erm^s transformants of each of these various $rpsL,erm$-marked dele-
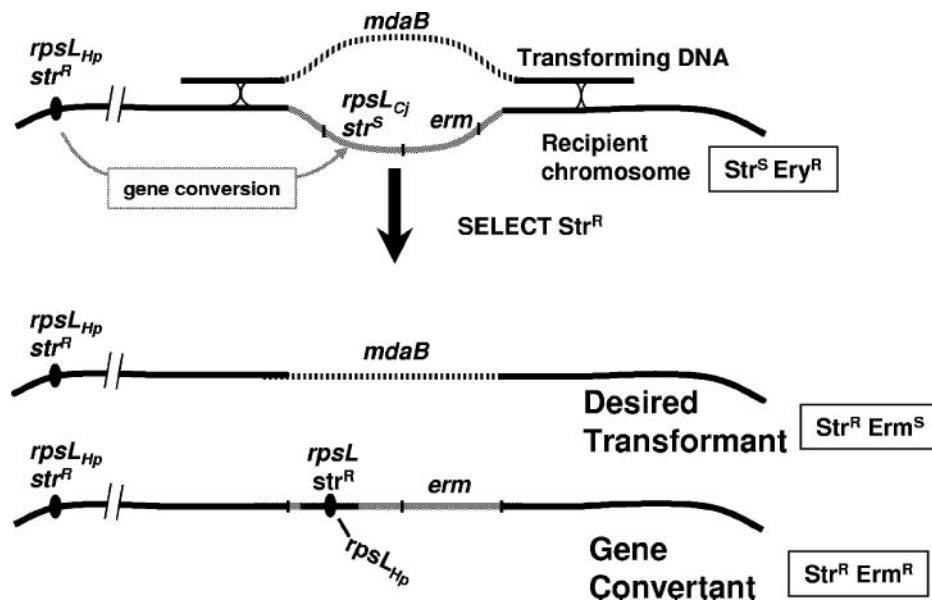

FIG. 3. Strategy for the use of $rpsL$ from $C. jejuni$ as a contraselectable determinant of streptomycin susceptibility in $H. pylori$. Diagrammed here is a recipient strain carrying an str^r allele of its normal ($H. pylori$) $rpsL$ gene and also the $rpsL,erm$ cassette that had (in an earlier transformation) replaced the $mdaB$ gene, now being transformed with genomic DNA from a strain with full-length $mdaB$, and acquisition of mdaB plus flanking sequences by crossing over in regions of sequence homology. This results in replacement of the $rpsL,erm$ cassette and a change from Erm^r to Erm^s in phenotype. Unwanted gene conversion events also result in an Str^r phenotype, but the Erm^r phenotype is retained.

tion strains verified the expected replacement of the *rpsL,erm* allele in each case.

DISCUSSION

We constructed a two-gene cassette containing an *erm* (resistance) gene for selection and a *C. jejuni rpsL* gene (dominant streptomycin susceptibility; ribosomal protein S12) that is 18% divergent from *H. pylori rpsL* for contraselection. Each *rpsL,erm*-marked allele was first made by transformation of a PCR product into one strain and then moved to other strains by transformation with genomic DNA from this first PCR-verified transformant. In the future such strains might equally be generated using PCR products to strictly limit the amount of DNA adjacent to the *rpsL,erm* cassette that is acquired during transformation. It would also eliminate any risk of the acquisition of unlinked genes (although this was not a major concern here because simultaneous transformation for two unlinked markers is rare).

Our results with four *rpsL,erm*-marked loci in four unrelated strains suggest that this cassette can be placed at any nonessential site in *H. pylori* strains of interest. Once inserted, other DNAs can be moved to that marked locus by transformation and homologous recombination. The desired transformants are identified simply by screening a relatively few Str^r colonies to identify those with the desired Erm^s phenotype. The yields of these replacements among selected Str^r colonies varied from ~17% to ~90%, depending on the gene targeted and the strain background. That this variation was seen with genomic DNAs from isogenic wild-type donor strains indicates that it is not due to DNA restriction. Rather, it may reflect local and genomewide effects on relative efficiencies of transformation versus gene conversion between related but divergent sequences. If further improvement in efficiency were needed, this could likely be achieved with a synthetic *rpsL* gene that is even more divergent from the *H. pylori rpsL* in DNA sequence (to further inhibit gene conversion), while exploiting the degeneracy of the code to ensure that it still encodes an *H. pylori*-like S12 protein.

In other experiments to date, we used this *rpsL,erm* cassette to replace point mutant alleles with wild-type alleles and vice versa, to replace full-length genes with unmarked deletion alleles, and to create a novel triple mutant allele of the *fur* regulatory gene (itself generated by PCR without cloning) for functional analyses of iron and pH-responsive transcriptional regulation. We anticipate additional applications for this cassette, including (i) the functional characterization of genes from divergent clinical isolates; (ii) altering key regulatory sites; (iii) engineering new domains or epitopes in surface-exposed or -secreted proteins; (iv) adding new genes to genomes of interest; and (v) deletion or other changes in some or all members of multigene families, unimpeded by the numbers of changes that might be needed.

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