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Stabilization of a Plasmid Coding for a Heterologous Antigen in *Salmonella enterica* Serotype Typhi Vaccine Strain CVD908-*htrA* by Using Site-Specific Recombination

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A gene cassette incorporating the *crs-rsd* site-specific recombination system from the *Salmonella enterica* subsp. *enterica* serovar Dublin virulence plasmid improved the inheritance in *S. enterica* serotype Typhi strain CVD908-*htrA* of a multicopy plasmid expression vector. Use of this recombination cassette may improve expression of heterologous antigens from multicopy plasmid expression vectors in attenuated bacterial vaccine strains.

A number of attenuated bacterial strains, including *Salmonella enterica* subsp. *enterica* serovar Typhi, are being developed as live oral vaccines (6). Recombinant DNA technology has enabled the expression of heterologous (or foreign) antigens from unrelated pathogens in *Salmonella* serovar Typhi, thus allowing the potential development of oral vaccines against other pathogens using attenuated *Salmonella* serovar Typhi as the carrier. The genes for the foreign antigen may be part of a multicopy plasmid expression vector. However, while such plasmid vectors may demonstrate stable inheritance in laboratory strains of *Escherichia coli*, they are often lost rapidly from *Salmonella* serovar Typhi vaccine strains in the absence of selection. This may not only adversely affect the outcome of an immune response to the foreign antigen following vaccination but also have repercussions during the manufacturing process when growth of the vaccine strain in fermentors will be required. In addition, the presence of antibiotic resistance determinants in a live attenuated vaccine may be considered unacceptable, as this could contribute to the spread of resistance. This has led to efforts to develop means of maintaining plasmids coding for foreign antigens in attenuated *Salmonella* vaccine candidates and to identify alternative selection methods (3, 4, 7, 11–13). The methods that most successfully maintain multicopy plasmids in *Salmonella* serovar Typhi vaccine strains depend on selection. However, selection does not stabilize inheritance of the plasmid, it merely kills all the bacteria from which the plasmid has been lost. With unstable plasmids, selection, while leading to the persistence of the plasmid in most cells, can result in the slow growth of the strain under selection and rapid plasmid loss when selection is removed. These factors may have contributed to the failure of some *Salmonella* serovar Typhi vaccine strains expressing heterologous antigens from multicopy plasmid expression vectors to generate satisfactory immune responses in volunteers (15). Here we show that the site-specific recombination system *crs-rsd* from the *Salmonella* serovar Dublin virulence plasmid significantly improves the inheritance, in the absence of any selection, of a plasmid vector that codes for *Helicobacter pylori* urease. In addition, when a transcriptional terminator and a kanamycin-selectable trait were included, very stable plasmid inheritance was achieved, despite the fact that no kanamycin selection was applied.

The plasmid pHUR3 has been described previously (10). It incorporates the *ureAB* genes from *Helicobacter pylori* expressed from the *htrA* gene promoter with the replication origin and the β-lactamase gene (*bla*) from pBR322 (Fig. 1). The inheritance of this plasmid was assayed in the live attenuated *Salmonella* serovar Typhi vaccine candidate CVD908-*htrA* (19). LB broth cultures of the strain supplemented with "aro mix" (40 μg/ml each of L-Phe and L-Trp, 10 μg/ml each of *p*-aminobenzoic acid and 2, 3-dihydroxybenzoic acid [final concentrations]) (8) and ampicillin at 200 μg/ml were grown overnight at 37°C with shaking. These cultures were used to inoculate volumes of fresh LB broth without antibiotic selection, and following incubation for 6 to 16 h, samples of the cultures were diluted and then spread on LB agar and on LB agar supplemented with ampicillin. The cultures were then used to inoculate additional volumes of fresh LB broth without antibiotic selection, and the process was repeated as described above. This process was continued for up to five additional passages. The numbers of colonies obtained on the LB agar and the LB agar supplemented with ampicillin after overnight incubation at 37°C were used to determine the percentage of cells that retained the plasmid and to estimate the average number of generations by using the formula $N_n = N_0 \times 2^n$, where N_n is the number of CFU/ml after n generations and N_0 is the number of CFU/ml at the start of the passage. Figure 2 shows that after 40 generations and in the absence of any selection, the proportion of bacterial cells that retained the pHUR3 plasmid was less than 35% in seven separate experiments.

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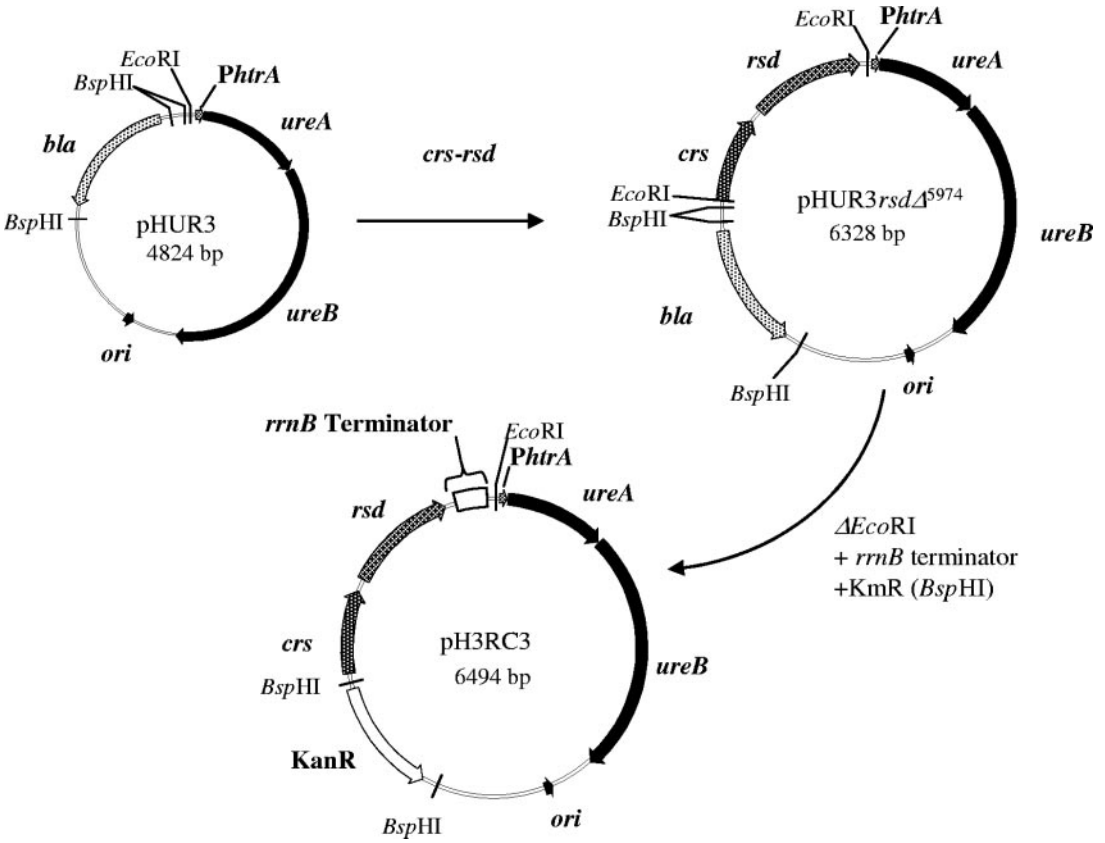

FIG. 1. Genetic maps of pHUR3, pHUR3*rsd*Δ^{5974}, and pH3RC3. Plasmid pHUR3*rsd*Δ^{5974} was made by inserting the *crs-rsd* cassette into pHUR3. Plasmid pH3RC3 was made from pHUR3*rsd*Δ^{5974} by removal of one EcoRI site, insertion of the *rrnB* transcriptional terminator into the remaining EcoRI site, and swapping the ampicillin resistance determinant for the kanamycin resistance determinant.

The *crs-rsd* site-specific recombination system present on the virulence plasmid pSDL2 of *Salmonella* serovar Dublin was shown to stabilize the inheritance in serovar Dublin of a plasmid derived from pACYC184 (9). We therefore constructed a *crs-rsd* cassette by amplifying the *crs* region from serovar Dublin using oligonucleotides 5′-GGCGAATTCCGGATGGCCT GTTGCAGGC and 5′-GCATCAGTAAAATGCACGAGC CTTTATACAGGGTTCAGAC and the *rsd* gene using oligonucleotides 5′-GGCGAATTCCCGGGTAAGTGTGGAT ATGTG and 5′-CTCGTGCATTTTACTGATGC with *Pfu* Turbo DNA polymerase (Invitrogen). The *crs-rsd* cassette was then generated in an overlap extension PCR (2) using the *crs* and *rsd* fragments as templates. The *crs-rsd* cassette fragment is flanked by EcoRI restriction sites which were used to ligate it into the EcoRI site of pHUR3 to generate plasmid pHUR3*rsd*Δ^{5974} (Fig. 1).

When pHUR3*rsd*Δ^{5974} was tested without selection in six independent experiments, it showed significantly greater stability of inheritance than pHUR3; at 40 generations, there was no significant loss of the plasmid in five of the six experiments (Fig. 2B). However, plasmid loss was still observed so that at 80 generations, there was no significant loss of the plasmid in only two of the six experiments. These results show that the *crs-rsd* cassette can significantly improve the inheritance of the pHUR3 plasmid when it is propagated in CVD908-*htrA*.

Plasmid pHUR3 is an expression vector in which the urease operon from *H. pylori*, *ureAB*, is expressed from the *Salmonella htrA* promoter. This ensures that expression of *ureAB* is induced when *Salmonella* serovar Typhi invades the host tissues, and this was intended to stabilize the plasmid and enhance immune responses against urease by ensuring induced expression only during in vivo growth (13). In addition, inappropriate expression of heterologous antigens may have a destabilizing influence on the inheritance of the plasmids by which they are encoded. It is therefore desirable to repress expression of heterologous antigens during in vitro growth to help maintain their encoding plasmid, while optimizing expression at the appropriate moment after vaccination. Krause and Guiney (9) demonstrated promoter activity in both directions from within the *crs* locus, and Western blot analysis of serovar Typhi strains harboring pHUR3*rsd*Δ^{5974} indicated that expression of UreAB was significantly higher than that from pHUR3 (not shown). This was also the case when the orientation of the *crs-rsd* cassette was reversed, confirming previous observations (9).

The 3′ region of the *rrnB* 5S gene of *E. coli* incorporates a strong transcriptional terminator. In order to counter the effects of the *crs-rsd* cassette on urease expression, a derivative of pHUR3*rsd*Δ^{5974} with this transcriptional terminator immediately upstream of the *htrA* promoter was constructed. To achieve this, a partial EcoRI digestion of pHUR3*rsd*Δ^{5974} was performed, the linearized fragment was isolated, the cohesive ends were filled using the Klenow fragment of DNA polymer-

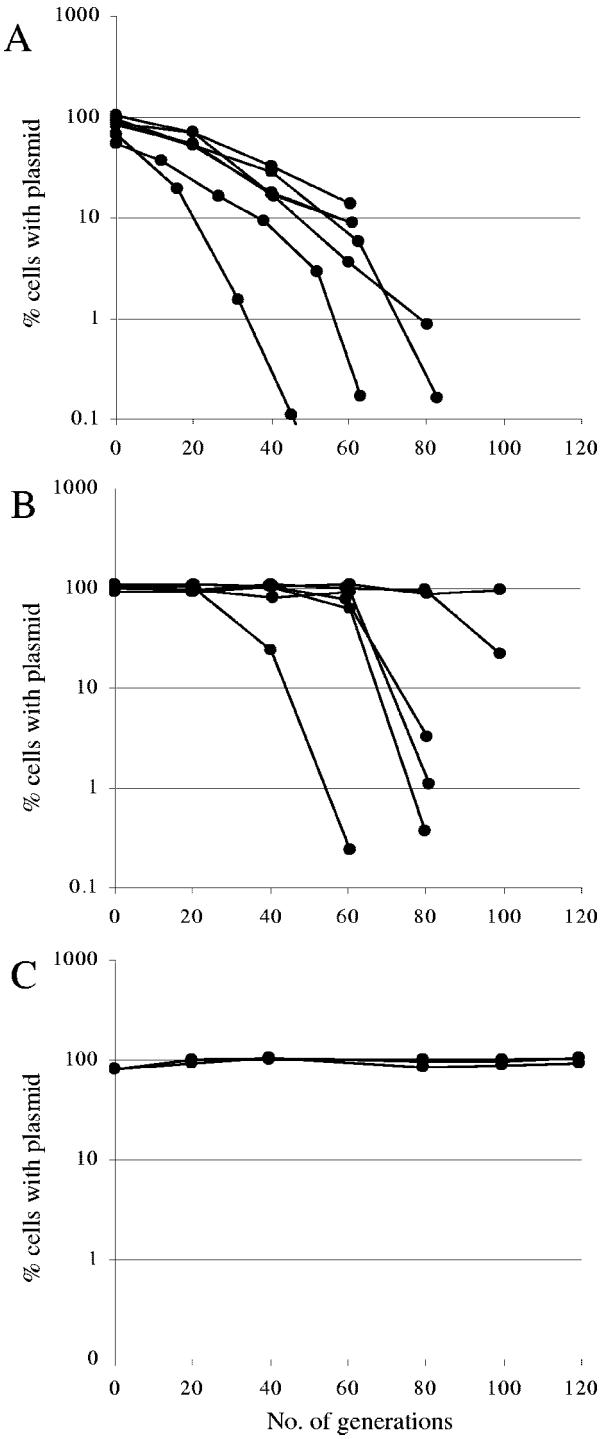

FIG. 2. Inheritance of pHUR3, pHUR3*rsd*Δ^{5974}, and pH3RC3 in *Salmonella* serovar Typhi strain CVD908-*htrA*. (A) pHUR3, results from seven separate experiments; (B) pHUR3*rsd*Δ^{5974}, results from six separate experiments; (C) pH3RC3, results from three separate experiments.

ase, and the treated DNA was ligated and transformed into *E. coli* DH5α. Transformants which harbored pHUR3*rsd*Δ^{5974} derivatives in which the EcoRI site between *bla* and *crs* had been disrupted were identified. One of these derivatives, pH3RC1, was chosen and linearized with EcoRI. The *rrnB* 5S gene transcriptional terminator was amplified using oligonucleotides 5′-GCCGAATTCACCGTATCTGTGGGGG GATG and 5′-GCGAAATTTCCCCATGCGAGAGTAGGG and *Pfu* Turbo DNA polymerase (Invitrogen), the resulting fragment was digested with restriction enzyme ApoI, and the fragment was then ligated to the linearized pH3RC1 to give plasmid pH3RC2. In addition, the β-lactamase gene, *bla*, of pH3RC2 was swapped with a kanamycin GenBlock (Amersham-Pharmacia) resistance determinant. To perform this, the kanamycin GenBlock determinant was amplified using oligonucleotides 5′-TCATCATGAACAATAAAACTGTCTGC and 5′-GCCTC ATGATCTGATCCTTCAACTCAGC, and the resulting fragment was ligated with pH3RC2 using BspHI restriction enzyme sites to give plasmid pH3RC3 (Fig. 1). This plasmid was introduced into CVD908-*htrA* and assayed for inheritance by using the method described above but substituting kanamycin for ampicillin in the starting culture that was used as a source of inoculum for the assay. In the absence of any selection, plasmid pH3RC3 was maintained without loss for 120 generations in three separate experiments (Fig. 2C).

The inheritance of multicopy plasmids is particularly unstable in *Salmonella* serovar Typhi, as illustrated by pHUR3. This has led to the development of several mechanisms for maintaining plasmids in serovar Typhi live vaccine candidates for delivery of heterologous antigens. However, these mechanisms are based on selection, which does not stabilize plasmid inheritance but kills all those bacteria that suffer plasmid loss. Thus far, clinical trials using *Salmonella* serovar Typhi as a carrier have failed to generate immune responses to plasmid-vectored heterologous antigens (1, 4, 18), and plasmid instability has been suspected as a cause (1). Galen et al. (7) were able to improve the inheritance of a multicopy plasmid in *Salmonella* serovar Typhi by using several mechanisms combined on one plasmid. However, even then, a small percentage of the bacteria showed plasmid loss after one culture passage, leading Galen et al. to question the rationale of using multicopy plasmids as vectors for heterologous antigens in serovar Typhi live vaccines. The data presented here demonstrate that the *crs-rsd-rrnB* terminator cassette, when combined with an appropriate selectable gene, can provide very stable inheritance of plasmids coding for foreign antigens in candidate vaccine strains of serovar Typhi, even in the absence of any selection. Generally, multimerization is a significant cause of multicopy plasmid loss in bacteria (17), and there are a number of reports describing the stabilizing influence of site-specific recombination systems on plasmid inheritance (5, 9, 14, 16, 20). Thus, the *crs-rsd-rrnB* terminator cassette described here may provide stability for a number of different plasmids in *Salmonella* serovar Typhi vaccine strains and enable the successful generation of immune responses in humans to plasmid-vectored heterologous antigens.

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