INFECTION AND IMMUNITY, Dec. 1999, p. 6424–6433 Vol. 67, No. 12
0019-9567/99/$04.00+0

Optimization of Plasmid Maintenance in the Attenuated Live Vector Vaccine Strain *Salmonella typhi* CVD 908-*htrA*†

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Received 6 July 1999/Returned for modification 20 August 1999/Accepted 24 September 1999

The broad objective of the research presented here is to develop a noncatalytic plasmid maintenance system for the stabilization of multicopy expression plasmids encoding foreign antigens in a *Salmonella typhi* live-vector vaccine strain such as CVD 908-*htrA*. We have enhanced the maintenance of expression plasmids at two independent levels. First, we removed dependence upon balanced-lethal maintenance systems that involve catalytic enzymes expressed from multicopy plasmids; we accomplished this through incorporation into expression plasmids of a postsegregational killing system based on the noncatalytic *hok-sok* plasmid addiction system from the antibiotic resistance factor pR1. We also included at least one naturally occurring plasmid partition function in our expression plasmids, which eliminates random segregation of these plasmids, thereby enhancing their inheritance and stability; to accomplish this, we incorporated either the *par* locus from pSC101, the *parA* locus from pR1, or both. We monitored the stability of optimized expression plasmids within CVD 908-*htrA* by quantitating expression of a variant of green fluorescent protein (GFPuv) by using flow cytometry. In this report, we demonstrate the utility of this novel plasmid maintenance system in enhancing the stability of our expression plasmids and go on to show that as the copy number of stabilized plasmids increases, the toxicity of GFPuv synthesis also increases. The implications of these observations for the rational design of immunogenic and protective bacterial live vector vaccines are discussed.

Bacterial live-vector vaccines represent a vaccine development strategy that offers exceptional flexibility. In this approach, genes that encode protective antigens of unrelated bacterial, viral, or parasitic pathogens are expressed in a live vector that carries the foreign antigens to the immune system, thereby eliciting an appropriate immune response. The attenuated *Salmonella typhi* vaccine strain CVD 908-*htrA* is a particularly attractive live vector in that it is well tolerated and elicits a broad immune response to *S. typhi* antigens, which includes intestinal soluble immunoglobulin A antibodies, serum immunoglobulin G antibodies, and cellular immune responses (47, 48). In addition, genetic methods have been developed to express foreign antigens within attenuated *S. typhi* vaccine strains, and a murine intranasal model has been developed as a practical animal model for examining the immunogenicity and protective efficacy of a wide variety of heterologous antigens within *S. typhi*-based live-vector vaccines, prior to initiating clinical trials (3, 16).

The efficacy of any bacterial live-vector vaccine rests with its ability to present sufficient foreign antigen to the human immune system to initiate the desired protective immune response. Controlled expression of heterologous antigens from multicopy expression plasmids represents one obvious solution for synthesis of high levels of antigen within live vectors. However, these plasmids may become unstable in vivo, resulting in the loss of foreign genes and a decrease in the intended immune response.

One method of enhancing the inheritance of expression plasmids by live vectors involves construction of a "balanced-lethal" system for plasmids expressing heterologous antigens (34). In a plasmid-based balanced-lethal system, plasmids replicating in the cytoplasm of the bacterium express a critical protein required by the bacterium to grow and replicate; loss of such plasmids removes the ability of the bacterium to express the critical protein and results in cell death. This phenomenon of plasmid loss during bacterial replication, which results in the death of any plasmidless bacterium, is also referred to as "postsegregational killing." Such a system has been successfully employed in *Salmonella typhimurium* and is based on expression of the *asd* gene encoding aspartate β-semialdehyde dehydrogenase (Asd) (15, 34). Asd is a critical enzyme involved in the synthesis of structural components essential for the formation of the cell wall in gram-negative bacteria. Therefore, loss of plasmids encoding such a critical enzyme would be lethal for any bacterium incapable of synthesizing Asd from the chromosome. Although the *asd* balanced-lethal system has been successfully employed in attenuated *S. typhimurium*-based live-vector strains for immunization of mice with a variety of prokaryotic and eukaryotic antigens (12, 28, 45), use of this method for stabilizing plasmids within attenuated *S. typhi* vaccine strains has, to date, been unsuccessful (47).

Here, we present the design and initial testing of a novel set of isogenic multicopy expression plasmids into which we have incorporated a noncatalytic postsegregational killing function, coupled with both active (14, 27) and passive plasmid partition functions (1, 31, 54), to provide a plasmid maintenance system designed to optimize expression of heterologous antigens within CVD 908-*htrA* for delivery to the human immune system. Since this method of improving plasmid maintenance

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involves no chromosomal mutagenesis of the live vector strain, in principle, such stabilized plasmids can be introduced into any live vector strain to improve the immunogenicity of heterologous antigens expressed, without additional genetic manipulations.

The approach is based on the use of the naturally occurring *hok-sok* postsegregational killing system residing on the R factor pR1 (19, 20). The *hok-sok* system is a two-component toxin-antitoxin system in which *hok* encodes a lethal pore-forming Hok protein. Synthesis of Hok is blocked by hybridization of a small antisense *sok* mRNA to *hok* mRNA, preventing translation and synthesis of Hok. However, *sok* mRNA is highly susceptible to degradation by nucleases, and its protective intracellular concentration must be maintained by constitutive transcription from resident plasmids carrying *hok-sok*. Therefore, bacteria that spontaneously lose such plasmids are postsegregationally killed because existing levels of the protective *sok* mRNA rapidly drop and levels of the more stable toxin-encoding *hok* mRNA quickly lead to Hok synthesis and cell death.

Inheritance of these expression plasmids has been enhanced through insertion of at least one partition function. A passive *par* locus from pSC101 (4, 31, 32, 52) was tested, in which no de novo partitioning proteins were encoded. In addition, the *parA* centromere-like active partitioning system from pR1 (14, 27) was investigated, since the combination of *hok-sok* and *parA* naturally occurs within pR1 and was not expected to present any compatibility complications. Plasmid maintenance was monitored by using flow cytometry to measure synthesis of a variant green fluorescent protein (GFPuv) test antigen within plasmid-bearing CVD 908-*htrA*. We show that although individual maintenance functions contribute to various degrees to the observed stability of expression plasmids, the highest levels of sustained synthesis of the test heterologous antigen GFPuv were detected from expression plasmids carrying the full complement of maintenance functions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All plasmid constructions were recovered in *Escherichia coli* DH5α (Gibco BRL). Construction of the *hok-sok* gene cassette used pR1 template DNA isolated from *E. coli* J53(pR1), a generous gift from James B. Kaper. The live vector *S. typhi* CVD 908-*htrA* is an auxotrophic derivative of the wild-type strain Ty2 with deletions in *aroC*, *aroD*, and *htrA* (48). All strains used in this work were grown in media supplemented with 2,3-dihydroxybenzoic acid (DHB) as previously described (16, 26). When grown on solid medium, plasmid-bearing strains of CVD 908-*htrA* were streaked from frozen (−70°C) master stocks onto 2× Luria-Bertani (LB) agar containing 20 g of Bacto Tryptone, 10 g of Bacto yeast extract, and 50 mM NaCl (2× LB agar) plus carbenicillin at a concentration of 50 μg/ml. Plates were incubated at 30°C for 24 to 36 h to obtain isolated colonies ~2 mm in diameter; strains were incubated at 30°C to minimize the toxicity of GFPuv expression in CVD 908-*htrA*.

For induction experiments involving comparison of the osmotically induced promoters P_{ompC1} and P_{ompC3}, strains were streaked from frozen master stocks onto 2× LB agar supplemented with DHB and carbenicillin and incubated for 36 h at 30°C. Induction conditions were adapted from those of Puente et al. (40) and Tartera and Metcalf (49); isolated colonies were pooled into 300 μl of nutrient broth supplemented with DHB and carbenicillin, from which 25 μl was inoculated into 25 ml of supplemented nutrient broth, with and without 150 mM NaCl, and incubated at 37°C at 250 rpm in an orbital shaker (Forma Scientific) for 24 h. Bacteria were then pelleted, resuspended in 1 ml of phosphate-buffered saline (PBS [pH 7.4]), and then diluted 1:1,000 into PBS for analysis by flow cytometry as described below.

For plasmid retention studies, all strains were streaked from frozen master stocks onto 2× LB agar supplemented with DHB and carbenicillin and incubated for 36 h at 30°C. Isolated colonies were pooled into 300 μl of 1× LB broth supplemented only with DHB, from which 25 μl was inoculated into 25 ml of 1× LB broth containing DHB and either 50, 150, or 300 mM NaCl; cultures were incubated at 37°C at 250 rpm for 24 h. Bacteria were then pelleted, resuspended in 1 ml of PBS (pH 7.4), and diluted 1:1,000 into PBS for analysis by flow cytometry.

Molecular genetic techniques. Standard techniques were used for the construction of the plasmids represented here (42). Unless otherwise noted, native *Taq* DNA polymerase (Gibco BRL) was used in PCRs. *S. typhi* strains were prepared for electroporation of recombinant plasmids after being harvested from Miller's LB broth (Gibco BRL) supplemented with DHB; after pelleting of bacteria, the cells were washed three times with one culture volume of sterile distilled water and resuspended in sterile distilled water to a final volume of 1/100 of the original culture volume. Electroporation of strains was performed in a Gene Pulser apparatus (Bio-Rad) set at 2.5 kV, 200 Ω, and 25 μF. Following electroporation, bacteria were repaired with SOC medium (Biofluids) and incubation at 37°C and 250 rpm for 45 min; bacteria were then plated on 1× LB medium containing DHB plus 50 μg of carbenicillin per ml, and incubated at 30°C for 24 h. Isolated colonies were then swabbed onto supplemented 2× LB medium and incubated at 30°C for 16 h. Frozen master stocks were prepared by harvesting bacteria into SOC medium without further supplementation and freezing at −70°C.

Construction of expression plasmids. (i) Construction of pJN1 and pJN2. All primers used in this work are listed in Table 1; essential plasmids created from these primers are listed in Table 2 to illustrate the flow of logic in designing the final isogenic expression plasmids. The expression plasmids constructed for these studies are composed of three basic cassettes encoding (i) expression of a heterologous antigen, (ii) a plasmid origin of replication, and (iii) selection and maintenance functions. To accomplish this, a basic replicon was constructed in which these cassettes were separated by unique restriction sites. pTET*nir*15 (Table 2) (38) was reengineered such that the *ori*E1 origin of replication and *bla* gene were separated by a unique *Spe*I site. Toward this end, an *ori*E1 cassette was synthesized by PCR with Vent polymerase with primers 1 and 2 and pCVD315 (17) as the template. The resulting 735-bp fragment carries engineered *Spe*I and *Bgl*II sites 5′ proximal to the promoter controlling transcription of RNA II, and an engineered *Avr*II site 675 bases from these sites. A separate PCR was carried out by using primers 3 and 4 to create a 1,234-bp *bla* cassette containing an engineered *Xba*I site 5′ proximal to the original *Eco*RI site. The products from these two PCRs were gel purified and used in an overlapping PCR with primers 1 and 4 to yield a final 1,916-bp *ori*E1-*bla* fragment which was self-ligated to create pJN1. The P_{nir15}-*toxC* fragment from pTET*nir*15 was excised as an *Eco*RI (partial digestion)-*Ava*I fragment, in which the *Ava*I terminus was polished, and inserted into the multiple cloning region from pSL1180 (9) cleaved with *Eco*RI and *Stu*I; this cassette was then reexcised as an *Eco*RI (partial digestion)-*Avr*II fragment and inserted into pJN1 cleaved with *Eco*RI-*Avr*II, creating pJN2 (Table 2).

(ii) Construction of pGFP*ompC*. To facilitate screening of a functional osmotically regulated P_{ompC} allele from *E. coli*, an *aphA-2* cassette was constructed, encoding resistance to the aminoglycosides neomycin and kanamycin (44). A PCR was carried out with primers 5 and 6 with the template pIB279 (5) to generate a 1,044-bp product, from which a promoterless 903-bp *aphA-2* *Bgl*II-*Nhe*I fragment was cleaved for replacement of a *Bgl*II-*Nhe*I *toxC* cassette encoding fragment C of tetanus toxin in pTET*nir*15. The anaerobically regulated P_{nir15} promoter was replaced with a 459-bp *Eco*RI-*Bgl*II P_{ompC} allele constructed with primers 7 and 8 with chromosomal template DNA from *E. coli* DH5α to create pK*ompC*. After confirming osmotic induction of P_{ompC} by examining the increase in resistance to kanamycin with increasing osmolarity, the *aphA-2* cassette was then replaced with a *gfpuv* gene encoding a prokaryotic codon-optimized GFPuv allele (Clontech) (13). The *gfpuv* gene was recovered by PCR with primers 9 and 10 with the template pGFPuv to generate a 751-bp *Bgl*II-*Nhe*I fragment, which was inserted into pK*ompC*, to generate pGFP*ompC*. Colonies were screened for functional GFPuv, and the brightest colonies were then examined for induction of fluorescence with increasing concentrations of NaCl. A P_{ompC1}-*gfpuv* cassette (see Results below) was cleaved from pGFP*ompC1* as an *Eco*RI-*Nhe*I fragment and inserted into a derivative of pJN2 cleaved with *Eco*RI-*Nhe*I to create pJJ4.

(iii) Construction of pNRB1, pGEN2, and pGEN3. Since it was intended that copy number not be influenced by transcription originating from promoters outside the origin of replication, it was necessary to ensure that all replication cassettes were flanked at both ends by transcription terminators. Because the origin and antigen cassettes of pJN2 are separated by the *trpA* terminator, it was only necessary to insert one additional terminator between the origin and *bla* cassettes.

To facilitate construction of additional plasmids later on, a *tetA*-T1T2 cassette was created. pYA292 (15) was first cleaved with *Hin*dIII and *Bgl*II, and the T1T2 terminator fragment was polished and inserted into the *Sma*I site of the pBluescript II KS (Stratagene) multiple cloning region; when the proper orientation was identified, this cassette was reexcised as a *Bam*HI-*Pst*I fragment and inserted into pIB307 (5) cleaved with *Bam*HI-*Pst*I, creating pJG14. It was later determined by sequence analysis that the cassette had undergone a deletion of approximately 100 bp, removing half of the T2 terminator.

Using pBR322 as a template, primers 11 and 12 were used to synthesize a 1,291-bp *tetA* *Bgl*II fragment. This *tetA* *Bgl*II fragment was then inserted into the *Bam*HI site of pJG14, such that transcription of the *tetA* gene is terminated at the T1T2 terminator, creating pJG14*tetA*. Finally, this *tetA*-T1T2 cassette was cleaved from pJG14*tetA* as an *Eco*RI-*Pst*I fragment in which the *Pst*I site was removed by polishing; the resulting fragment was inserted into pJJ4 cleaved with *Spe*I, polished, and recleaved with *Eco*RI to replace the *bla* cassette and create pNRB1.

TABLE 1. Primers used in construction of the plasmid cassettes

Primer no.	Sequence[a]	Cassette created	GenBank accession no.	Template	Source or reference
1	5′-GCAGGAAAGAACATGTGAG**CCTAGG**GCCAGCAAAAGGCCAGGAAC-3′	*ori*E1	J01749	pCVD315	17
2	5′-CATGACCAAAATCCCTTA**ACTAGT**GTTTT**AGATCT**ACTGAGCGTCAGACCCCG-3′	*ori*E1	J01749	pCVD315	
3	5′-CGGGGTCTGACGCTCAGT**AGATCT**AAAAC**ACTAGT**TAAGGGATTTTGGTCATG-3′	*bla*	J01749	pCVD315	
4	5′-GCTGTCAAACATGA**GAATTCTAGA**AGACGAAAGGGCCTCGTGATACGCC-3′	*bla*	J01749	pCVD315	
5	5′-ACAGCCTGCAGACAGATCTTGACAGCTGGATCGCACTCTGGTATAATTGGGAAGCCCTGCAAAG-3′	*aphA-2*	V00618	pIB279	5
6	5′-CGAAGCCCAACCTTTCATAGAA**GCTAGC**GGT**GGATCC**GAAATCTCGTGATGGCAGGTTG-3′	*aphA-2*	V00618	pIB279	
7	5′-AACAAGCGTTATAG**GAATTC**TGTGGTAGCA-3′	P_{ompC}	K00541	*E. coli* DH5α	Gibco BRL
8	5′-ACTTTCATGTTATTAA**AGATCT**GTTATATG-3′	P_{ompC}	K00541	*E. coli* DH5α	
9	5′-**AGATCT**TAATCATCCAC*AGGAGG*CTTTCTG*ATG*AGTAAAGGAGAAGAACTTTTCACTGG-3′	*gfpuv*	U62636	pGFPuv	13
10	5′-**GCTAGC**TCATTATTTGTAGAGCTCATCCATGC-3′	*gfpuv*	U62636	pGFPuv	
11	5′-**AGATCTGAATTCTAGA**TCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCG-3′	*tetA*	J01749	pBR322	2
12	5′-**AGATCT**TATCAGGTCGAGGTGGCCCGGCTCCATGCACCGCGACGCAACGCG-3′	*tetA*	J01749	pBR322	
13	5′-CGC**GAATTCTCGAG**ACAAACTCCGGGAGGCAGCGTGATGCGGCAACAATCACACGGATTTC-3′	*hok-sok-tetA*	X05813	pR1	19, 24
14	5′-ATGAGCGCATTGTTAGATTTCATTTTTTTTTCCTCCTTATTT**TCTAGA**CAACATCAGCAAGGAGAAAGG-3′	*hok-sok-tetA*	J01749, X05813	pR1	
15	5′-CCTTTCTCCTTGCTGATGTTG**TCTAGA**AAATA*AGGAGG*AAAAAAAAA*ATG*AAATCTAACAATGCGCTCAT-3′	*hok-sok-tetA*	X05813, J01749	pBR322	2
16	5′-GCTACATTTGAAGAGATAAATTGCACT**GGATCC**TAGAAATATTTTATCTGATTAATAAGATGATC-3′	*ori*15A	X06403	pACYC184	43
17	5′-CGGAGATTTCCTGGAAGATG**CCTAGG**AGATACTTAACAGGGAAGTGAGAG-3′	*ori*15A	X06403	pACYC184	
18	5′-GTCTGCCGGATTGCTTATCCTGGC**GGATCC**GGTTGACAGTAAGACGGGTAAGCCTGTTGAT-3′	*par*	X01654	pSC101	33
19	5′-AGGCTTAAGTAGCACCCTCGCA**AGATCT**GGCAAATCGCTGAATATTCCTTTTGTCTCCGAC-3′	*par*	X01654	pSC101	
20	5′-GAGGGCGCCCCAGCTGGCAAT**TCTAGACTCGAG**CACTTTTGTTACCCGCCAAACAAAACCCAAAAACAAC-3′	*aphA-2–parA*	V00618, X04268	pR1	19, 24
21	5′-AGAAGAAAAATC**GAATTC**CAGCATGAAGAGTTTCAGAAAATGACAGAGCGTGAGCAAGTGC-3′	*aphA-2–parA*	X04268	pR1	
22	5′-GTTGTTTTTGGGTTTTGTTTGGCGGGTAACAAAAGTG**CTCGAGTCTAGA**ATTGCCAGCTGGGGCGCCCTC-3′	*aphA-2–parA*	X04268, V00618	pIB279	5
23	5′-CGAAGCCCAACCTTTCATAGAA**ACTAGT**GGTGGAATCGAAATCTCGTGATGGCAGGTTG-3′	*aphA-2–parA*	V00618	pIB279	

[a] Relevant restriction sites are designated in boldface, underlined, and referred to in the text; ribosome binding sites and start codons are designated in italics.

TABLE 2. Selected plasmids used in this work

Plasmid	Size (kb)	Relevant genotype	Source or reference
pTET*nir*15	3.7	*ori*E1 *toxC bla*	38
pJN1	1.9	*ori*E1 *bla*	This work
pJN2	3.4	*ori*E1 *toxC bla*	This work
pGFPuv	3.3	pUC19*ori gfpuv bla*	Clontech
pGFP*ompC*	3.5	*ori*E1 *gfpuv bla*	This work
pNRB1	3.5	*ori*E1 *gfpuv tetA*	This work
pGEN2	4.2	*ori*E1 *gfpuv tetA hok-sok*	This work
pGEN3	4.1	*ori*15A *gfpuv tetA hok-sok*	This work
pJN5	3.1	*ori*E1 *gfpuv bla*	This work
pJN6	3.7	*ori*E1 *gfpuv bla hok-sok*	This work
pJN7	4.1	*ori*E1 *gfpuv bla hok-sok par*	This work
pJN8	5.4	*ori*E1 *gfpuv bla hok-sok parA*	This work
pGEN51	3.6	*ori*E1 *gfpuv bla*	This work
pGEN71	4.2	*ori*E1 *gfpuv bla hok-sok*	This work
pGEN84	4.5	*ori*E1 *gfpuv bla hok-sok par*	This work
pGEN183	5.9	*ori*E1 *gfpuv bla hok-sok parA*	This work
pGEN211	6.2	*ori*E1 *gfpuv bla hok-sok par parA*	This work
pGEN91	3.5	*ori*15A *gfpuv bla*	This work
pGEN111	4.1	*ori*15A *gfpuv bla hok-sok*	This work
pGEN121	4.5	*ori*15A *gfpuv bla hok-sok par*	This work
pGEN193	5.8	*ori*15A *gfpuv bla hok-sok parA*	This work
pGEN222	6.2	*ori*15A *gfpuv bla hok-sok par parA*	This work

The noncatalytic postsegregational killing function to be incorporated into the plasmid maintenance systems of the expression plasmids described here was the *hok-sok* locus, from the multiple drug resistance R factor pR1. Initial attempts at recovering the *hok-sok* locus after PCR were unsuccessful. It was therefore necessary to use overlapping PCR to generate a cassette in which *hok-sok* was transcriptionally fused to a promoterless *tetA* gene such that transcription originating from the *hok* promoter would continue into *tetA* and result in a transcript encoding both Hok and resistance to tetracycline. pR1 plasmid DNA was purified from *E. coli* J53(pR1) in which pR1 encodes resistance to both carbenicillin and chloramphenicol. A 640-bp *hok-sok* fragment was synthesized by using primers 13 and 14; a promoterless 1,245-bp *tetA* fragment was recovered in a separate PCR by using primers 15 and 12 with pNRB1 as the template. The products from these two PCRs were then used in an overlapping PCR with primers 12 and 13 to yield the final 1,816-bp *hok-sok-tetA* fragment. This fragment was inserted as an *Eco*RI-*Sph*I fragment into pNRB1 cleaved with *Eco*RI-*Sph*I, regenerating the *tetA* gene and creating pGEN1.

Two isogenic plasmids were then constructed, differing only in copy number, from which all further expression plasmids would be derived. The *Bgl*II-*Avr*II origin of replication cassette of pGEN1 was replaced by a *Bgl*II-*Avr*II *ori*E1 cassette from pJN2 to generate pGEN2. In addition, an *ori*15A replication cassette was synthesized by PCR by using primers 16 and 17 with pACYC184 template to generate a 629-bp *Bam*HI-*Avr*II fragment, which was inserted into pGEN2 cleaved with *Bgl*II-*Avr*II to create pGEN3.

(iv) Construction of pJN5, pGEN51, and pGEN91. The principle set of isogenic expression plasmids, to which individual elements of a plasmid maintenance system were sequentially added, was composed of pGEN51 (containing *ori*E1) and pGEN91 (containing *ori*15A). The basic replicon from which these two plasmids were constructed was pJN5, which was assembled by cleaving the P_{ompC}-*gfpuv* cartridge as an *Eco*RI-*Nhe*I fragment from pGFP*ompC* to replace the P_{nir15}-*toxC* cassette of pJN2. Construction of pGEN51 was then accomplished by removal of the replication cassette from pGEN2 as a *Bam*HI fragment and replacement of the origin of replication within pJN5 digested with *Bgl*II and *Bam*HI, thereby regenerating the *gfpuv* gene. Construction of pGEN91 was accomplished in an identical manner by excision of the origin cassette from pGEN3 as a *Bam*HI fragment and insertion into pJN5 (Fig. 1 and Table 2).

(v) Construction of pJN6, pGEN71, and pGEN111. The *hok-sok* locus was then excised as an *Xba*I-*Sal*I fragment from pGEN2 and inserted into pJN5 cleaved with *Xba*I and *Sal*I, again regenerating the *gfpuv* gene to create pJN6. Construction of pGEN71 and pGEN111 was then carried out exactly as described for pGEN51 and pGEN91 by insertion into pJN6 of origin cassettes as *Bam*HI fragments from pGEN2 and pGEN3, respectively (Fig. 1 and Table 2).

(vi) Construction of pJN7, pGEN84, and pGEN121. Construction of *ori*E1 and *ori*15A expression plasmids containing a plasmid maintenance system, composed of both a postsegregational killing system and at least one partition function, was first attempted by using the *par* function from pSC101. A 377-bp *Bam*HI-*Bgl*II fragment was synthesized with primers 18 and 19 with pSC101 template DNA; this fragment was inserted into pJN6 cleaved with *Bgl*II to create pJN7. As in the constructions above, origin cassettes from pGEN2 and pGEN3 were then excised as *Bam*HI fragments and inserted into pJN7 digested with *Bgl*II and *Bam*HI to create pGEN84 and pGEN121.

(vii) Construction of pJN8, pGEN183, pGEN193, pGEN211, and pGEN222. The final expression plasmids were constructed by introduction of the *parA* active partitioning locus from pR1. As with *hok-sok*, initial attempts at recovering the *parA* locus after PCR were unsuccessful. It was necessary to use overlapping PCR to generate an *aphA-2–parA* cassette, in which *aphA-2* and *parA* were divergently transcribed and separated by *Xba*I and *Xho*I sites, to enable subcloning of the *parA* locus. A 1,737-bp *parA* fragment was synthesized by using primers 20 and 21 with pR1 template; a 1,076-bp *aphA-2* fragment was recovered in a separate PCR by using primers 22 and 23 with pIB279 as the template. The products from these two PCRs were then used in an overlapping PCR with primers 21 and 23 to yield the final 2,743-bp *aphA-2–parA* fragment. This fragment was inserted as a 2,703-bp *Eco*RI-*Spe*I fragment into pJN6. The *parA* cassette was then reexcised as an *Xho*I fragment and inserted again into pJN6 cleaved with *Xho*I, regenerating the *gfpuv* gene and creating pJN8.

Plasmids carrying a plasmid maintenance system, composed of the postsegregational killing *hok-sok* function and *parA*, were constructed by excision of *ori*E1 and *ori*15A *Bam*HI-*Spe*I cassettes from pGEN51 and pGEN91, respectively, and insertion into pJN8 cleaved with *Bam*HI and *Spe*I, regenerating *gfpuv* and creating pGEN183 and pGEN193, respectively. Plasmids containing the full complement of *hok-sok*, *par*, and *parA* maintenance functions were constructed by insertion of *par*-containing origin cassettes as *Bam*HI-*Spe*I cassettes from pGEN84 and pGEN121 into pJN8 cleaved with *Bam*HI and *Spe*I, again regenerating *gfpuv* to create pGEN211 and pGEN222, respectively.

Flow cytometry. Quantitation of GFPuv and plasmid maintenance were analyzed by measuring the fluorescence of plasmid-bearing live vectors by using an Epics Elite ESP flow cytometer/cell sorter system (Coulter) with the argon laser exciting bacteria at 488 nm and emissions detected at 525 nm. Twenty-five-milliliter 1× LB cultures grown as described above were pelleted, and bacteria were resuspended in 1 ml of PBS. Cells were then diluted 1:1,000 in PBS prior to determination of viable counts and flow analysis. Forward versus side light scatter, measured with logarithmic amplifiers, was used to gate on bacteria. A minimum of 30,000 events were acquired from each sample at a collection rate of approximately 3,500 events per second. Mean fluorescence intensity for a given bacterial population was determined by using the Epics Elite Software Analysis Package. The fluorescence for plasmidless *S. typhi* CVD 908-*htrA* and *E. coli* DH5α strains was quantitated to establish that autofluorescence from either host strain was negligible and that fluorescence measured for plasmid-bearing strains was directly related to synthesis of GFPuv.

RESULTS

Rationale for construction of the expression plasmids. Although balanced-lethal plasmid stabilization systems based on expression of Asd have been created to maintain plasmids within *Salmonella*, a potential limitation of the *asd* system is its reliance on synthesis of an enzyme with catalytic activity. Since complementation with only a single copy of *asd* is sufficient to remove auxotrophy (15), it is not clear why all copies of a multicopy expression plasmid should remain stable and maintain maximum gene dosage, especially if they encode an especially problematic antigen which inhibits growth of the bacterium. Another potential limitation of the *asd* system is that it does not enhance the inheritance of resident plasmids, which continue to segregate randomly with or without the presence of the *asd* system. Therefore, if resident expression plasmids carrying *asd* genes are inherently unstable for some undetermined reason, they will be lost regardless of the requirement of the bacterium for Asd. Here, we present the design and initial testing of a set of isogenic multicopy expression plasmids into which we have incorporated a noncatalytic postsegregational killing function, coupled with both active (14, 27) and passive plasmid partition functions (1, 31, 54), to provide a plasmid maintenance system designed to optimize expression of heterologous antigens within CVD 908-*htrA* for delivery to the human immune system.

Two series of isogenic expression plasmids were constructed for use in *E. coli* and *Salmonella*, with expected copy numbers of ~60 copies per chromosomal equivalent (from pAT153, carrying a derivative of *ori*E1 [11, 38, 50]) or ~15 copies per equivalent (derived from pACYC184 and carrying *ori*15A [10, 25]). Each plasmid comprises three basic cassettes encoding the origin of replication; a plasmid selection cassette encoding

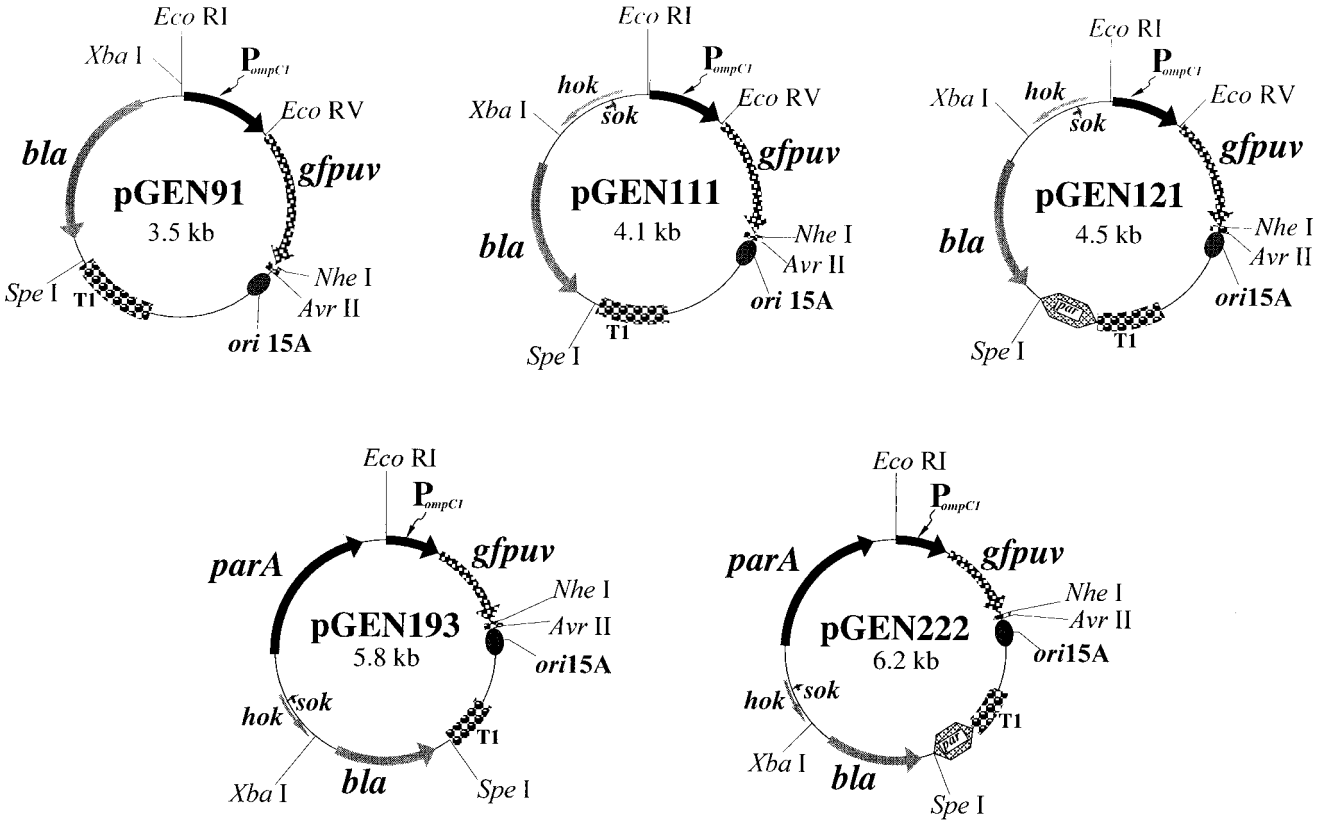

FIG. 1. Genetic maps of a representative set of isogenic expression plasmids, carrying an *ori*15A origin of replication, which differ only in the introduction of increasingly complex combinations of maintenance functions. The plasmids pGEN111, pGEN121, pGEN193, and pGEN222 contain maintenance functions encoded by the *hok-sok*, *hok-sok* plus *par*, *hok-sok* plus *parA*, and *hok-sok* plus *par* plus *parA* loci, respectively. Other expression plasmids were constructed by replacing each *ori*15A origin of replication cassette (copy number, ~15) with an *ori*E1 origin (copy number, ~60); a list of the plasmids used in this work is given in Table 2. The key restriction sites shown represent unique sites in the expression plasmids. P_{ompC1}, modified osmotically controlled *ompC* promoter from *E. coli*; *gfpuv*, gene encoding the prokaryotic codon-optimized GFPuv; T1, transcriptional terminator from the *rrnB* rRNA operon of *E. coli*; *par*, passive partitioning system from pSC101; *bla*, β-lactamase gene conferring resistance to carbenicillin; *hok-sok*, postsegregational killing locus from the multiple antibiotic resistance R plasmid pR1; *parA*, active partitioning system from pR1.

β-lactamase, which confers resistance to carbenicillin; and a heterologous antigen expression cassette. A representative set of expression plasmids containing an *ori*15A origin of replication is shown in Fig. 1. The heterologous antigen cassette of the basic expression vector pGEN91 is composed of individual cassettes encoding an inducible promoter to control transcription of the heterologous antigen cassette, which for the work presented here encodes the test antigen GFPuv. Components of a plasmid maintenance system were then systematically inserted into pGEN91 to assess any individual or synergistic influence of these functions on plasmid stability in the presence and absence of selection. A complete plasmid maintenance system will be defined here as being composed of a postsegregational killing function and both a passive and an active plasmid-partitioning function. For the expression plasmids reported here, the postsegregational killing function is represented by the *hok-sok* locus; this locus was inserted into pGEN91 to create pGEN111 and ensure that flanking transcription from surrounding loci, such as the antigen and selection cassettes, was divergent and would not significantly disturb the wild-type transcription levels which control the lethality of this locus. We also examined the effects of both passive and active partitioning loci on expression plasmid stability and synthesis of GFPuv. We inserted the *par* passive partition locus between the origin of replication and selection cassettes (Fig. 1, pGEN121). Interestingly, it was noted that the orientation of the *par* locus enhanced synthesis of GFPuv on solid medium when inserted in the natural orientation found within *ori*101 of pSC101, and this orientation was adopted for all of the expression plasmids. The active partitioning locus chosen for this work was the *parA* locus from the same pR1 resistance plasmid from which *hok-sok* was adapted; it was expected that the compatibility of these two loci within pR1 would be maintained within our expression plasmids. Again, to preserve natural transcription levels and regulation within this locus, the cassette was positioned within an area of the expression plasmids such that flanking transcription progressed away from *parA* (Fig. 1, pGEN193 and pGEN222).

Osmotic control of P_{ompC}. It was intended that any promoter controlling transcription of a heterologous gene be responsive to an environmental signal of biological relevance. For the expression plasmids described here, an *ompC* promoter cassette (P_{ompC}) from *E. coli* was used, which is induced by increases in osmolarity. Construction of this cassette was based on the sequence of P_{ompC} published by Norioka et al. (37) and was engineered to control expression of a test antigen cassette containing the *gfpuv* allele encoding GFPuv; this P_{ompC}-*gfpuv* cassette was inserted into a derivative of pBR322 to create pGFP*ompC*. During the visual screening of *E. coli* DH5α(pGFP*ompC*) colonies subilluminated with UV light,

TABLE 3. Comparison of induction of P_{ompC1} and P_{ompC3}, controlling expression of GFPuv, within the host strains *E. coli* DH5α and CVD 908-*htrA*

Strain	Induction result with:				Induction ratio[c]
	Low osmolarity		150 mM NaCl		
	OD_{600}[a]	Mean fluorescence intensity[b]	OD_{600}	Mean fluorescence intensity[b]	
DH5α	0.61	0.3	0.95	0.3	NA[d]
DH5α(pGFP*ompC1*)	0.56	4.5	0.72	7.7	1.7
DH5α(pGFP*ompC3*)	0.58	1.8	0.73	4.2	2.4
CVD 908-*htrA*	0.58	0.3	0.65	0.3	NA
CVD 908-*htrA*(pGFP*ompC1*)	0.60	5.4	0.54	23.4	4.4
CVD 908-*htrA*(pGFP*ompC3*)	0.54	2.6	0.53	17.1	6.7

[a] OD_{600}, optical density at 600 nm.
[b] All standard error values are less than 0.1.
[c] Defined as the ratio of mean fluorescent intensity measured after induction with 150 mM NaCl divided by the basal level of mean fluorescent intensity measured at low osmolarity.
[d] NA, not applicable.

one very brightly fluorescing colony and another representative fluorescent colony were chosen for further study, designated clones 1 and 3, respectively. Upon purification of the plasmids involved, it was determined that clone 1 contained a plasmid that no longer carried a *Bgl*II site separating P_{ompC} and *gfpuv*, while clone 3 carried the expected *Bgl*II site. We examined the induction of GFPuv expression when clones 1 and 3 were grown on nutrient agar in the presence or absence of NaCl and determined by visual inspection that clone 3 displayed very little fluorescence when grown in the absence of NaCl but fluoresced brightly when plated on medium containing 300 mM NaCl (data not shown). Clone 1, however, had a higher background level of fluorescence when uninduced, but fluoresced intensely when induced with 300 mM NaCl. To rule out mutations within the *gfpuv* gene which might affect fluorescence, we replaced P_{ompC} from clone 1 with P_{ompC} from clone 3 and confirmed the expected decrease in fluorescence as judged by subillumination (data not shown). We therefore concluded that differences in observed fluorescence were controlled by two genetically distinct versions of the P_{ompC} promoter, which we designate as P_{ompC1} (higher transcription levels with less osmotic control) and P_{ompC3} (moderate transcription levels but more responsive to osmolarity); we have designated the plasmids containing these expression cassettes as pGFP*ompC1* and pGFP*ompC3*, respectively.

To quantify the differences between induced and uninduced expression of *gfpuv* controlled by P_{ompC1} and P_{ompC3}, GFPuv synthesis was monitored within both *E. coli* DH5α and *S. typhi* CVD 908-*htrA* by flow cytometry. This powerful technique has the unique advantage of allowing rapid measurement of GFPuv expression within large numbers of individual bacteria, as well as accurately determining the intensity of fluorescence due to GFPuv synthesis within each bacterium analyzed. As summarized in Table 3, the basal level of expression for the P_{ompC1}-*gfpuv* cassette is 2.5-fold higher than that for the P_{ompC3}-*gfpuv* cassette, when expressed in DH5α, and 2.1-fold higher when expressed within CVD 908-*htrA*; however, the basal level of fluorescence detected for synthesis of GFPuv never exceeded a mean fluorescent intensity of 5.4, regardless of host background. If we define the induction ratio as the ratio of mean fluorescent intensity measured after induction divided by the basal level of mean fluorescent intensity, it was observed that when induced with 150 mM NaCl, P_{ompC1} and P_{ompC3} displayed within DH5α induction ratios of 1.7 and 2.4, respectively. Surprisingly, the induction ratio for P_{ompC1} when measured in CVD 908-*htrA* was 4.4, and P_{ompC1} produced a maximum mean fluorescence intensity of 23.4 for these experiments. Although the induction ratio for P_{ompC3} within CVD 908-*htrA* was 6.7, the mean fluorescence intensity of 17.1 was lower than that measured for P_{ompC1}.

Since P_{ompC3} was noted to possess the intended 3′-terminal *Bgl*II site, which was not detected for P_{ompC1}, we determined the nucleotide sequence for P_{ompC1} to perhaps detect point mutations which might explain the strength of P_{ompC1}. The only differences identified were located at the 3′ terminus of the cassette. The intended sequence within this region was 5′-. . .catataacAGATCTtaatcatccacAGGAGGatatctgATG-3′. (From left to right, uppercase denotes the *Bgl*II site, ribosome binding site, and GFPuv start codon, respectively.) The actual sequence proved to be 5′-. . .catataacAGATC**GATCT**taa**A**catc cacAGGAGG**A**t**A**tctgATG-3. (Inserted or changed bases are denoted by underlined bold uppercase letters.) These changes detected within the *ompC1* promoter sequence are apparently responsible for increasing the observed strength of P_{ompC1} by an unknown mechanism, since neither the basic *ompC* promoter sequence nor the optimized ribosome binding site has been spontaneously altered.

These data clearly show that when driving expression of *gfpuv* within the live-vector strain CVD 908-*htrA*, P_{ompC1} and P_{ompC3} are inducible with increasing osmolarity, although the basal level of transcription is still noteworthy in both cases. It appears that P_{ompC1} is the strongest of the two osmotically responsive *ompC* promoters; P_{ompC1} was therefore chosen for synthesis of the widest possible range of heterologous test antigen and the effects of such synthesis on plasmid stability.

Stability of expression plasmids in the absence of selection. Since the broad objective of the research presented here is to develop a noncatalytic plasmid maintenance system to enhance the stability of multicopy expression plasmids encoding foreign antigens within CVD 908-*htrA*, we initiated experiments to monitor plasmid stability by quantitating expression of GFPuv by flow cytometry when strains were passaged in the absence of antibiotic selection. These experiments were designed to address three fundamental questions. (i) What is the effect of copy number on the stability of plasmids expressing GFPuv? (ii) What is the effect of the induction level of P_{ompC1} on the stability of plasmids encoding a heterologous antigen such as GFPuv? (iii) How do the *hok-sok*, *par*, and *parA* maintenance

TABLE 4. Stability within CVD 908-*htrA* of *ori*15A replicons, containing plasmid maintenance systems of increasing complexity, grown without selection and in the presence of increasing osmolarity[a]

Strain or plasmid	Stability result with NaCl concn:								
	50 mM			150 mM			300 mM		
	OD_{600}[b]	% Fluorescing bacteria	Mean fluorescence intensity[c]	OD_{600}	% Fluorescing bacteria	Mean fluorescence intensity[c]	OD_{600}	% Fluorescing bacteria	Mean fluorescence intensity[c]
Strain									
CVD 908-*htrA*	0.98	100	0.6	1.11	100	0.6	1.12	100	0.6
Plasmids									
pGEN91	1.00	13.2	28.6	1.17	11.4	42.9	1.26	10.9	65.5
pGEN111	1.26	47.4	51.8	1.17	28.9	93.6	1.12	42.4	65.1
pGEN121	1.01	80.5	53.3	1.20	73.8	74.0	1.15	56.7	105.3
pGEN193	1.11	71.4	50.9	1.24	65.2	64.7	1.22	53.7	90.8
pGEN222	1.01	96.8	52.1	1.28	93.3	67.8	1.13	95.3	89.2

[a] These data are represented by histograms in Fig. 2.
[b] OD_{600}, optical density at 600 nm.
[c] All standard error values are <0.6.

functions affect plasmid retention, both as individual components and synergistically?

Initial flow cytometry experiments were carried out in which CVD 908-*htrA* carried isogenic replicons with various maintenance functions and either the *ori*E1 or *ori*15A origin of replication. (Figure 1 depicts the isogenic series of *ori*15A replicons.) It was quickly determined that replicons carrying the higher-copy-number *ori*E1 origins with maintenance functions were very unstable, even when strains were grown in the presence of antibiotic selection. Flow cytometry results indicated that even when cultured in the presence of carbenicillin, the percentage of the bacterial populations no longer expressing detectable GFPuv ranged from approximately 50% for constructs carrying either *hok-sok* or *hok-sok* plus *par* to 62% for constructs with *hok-sok* plus *par* plus *parA*. Since replicons carrying an *ori*E1 origin clearly did not allow for optimal synthesis of the heterologous GFPuv test antigen within the majority of a growing population of live-vector bacteria, this series of expression plasmids was not examined further.

Maintenance of expression plasmids containing an *ori*15A origin of replication was then examined. Results for CVD 908-*htrA* harboring a particular expression plasmid and passaged for 24 h in the absence of selection are listed in Table 4; histograms representing these data are shown in Fig. 2. In general, as osmolarity increased and induction of P_{ompC1} rose, the percentage of the live-vector population expressing GFPuv decreased; nevertheless, the mean level of fluorescence intensity increased as expected. For example, in the presence of 50 mM NaCl, 80.5% of a population of CVD 908-*htrA*(pGEN121) expressed GFPuv with a mean fluorescence intensity of 53.3; as the concentration of NaCl increased to 300 mM, only 56.7% of the population expressed GFPuv, but the mean fluorescence intensity jumped to 105.3. However, it is notable that for strains carrying pGEN222 with a complete plasmid maintenance system (i.e., *hok-sok* plus *par* plus *parA*), the percentage of the population expressing the heterologous antigen remained at approximately 95% regardless of induction, while the mean fluorescence intensity increased from 52.1 (50 mM NaCl) to 89.2 (300 mM NaCl). It was noted that upon passage of these strains for an additional 24 h in the absence of antibiotic selection, less than 5% of bacteria continued to express functional GFPuv. Streaks of these cultures onto solid medium, prior to flow analysis, indicated that nonfluorescing bacteria remained viable; this was confirmed when nonfluorescing bacteria were sorted and shown by plating to be sensitive to antibiotic and nonfluorescing when irradiated with UV light, indicating loss of resident plasmids.

Taken together, these data suggest that as copy number is reduced, the apparent stability of resident plasmids and proficiency of a live vector to synthesize a heterologous antigen such as GFPuv increase. However, as the induction of P_{ompC1} and concomitant production of the heterologous antigen increase, the percentage of a growing population remaining capable of synthesizing antigen can be dramatically reduced; as plasmid maintenance functions accumulate within a given plasmid, apparent stability and antigen synthesis are restored.

DISCUSSION

The broad objective of the research presented here is to develop a plasmid maintenance system for the stabilization of multicopy expression plasmids encoding foreign antigens in an *S. typhi* live-vector vaccine strain, without additional modification of the chromosome. Attempts were made to enhance the maintenance of expression plasmids at two independent levels. First, dependence upon balanced-lethal maintenance systems that involve catalytic enzymes expressed from multicopy plasmids was removed; this was accomplished through incorporation into expression plasmids of a postsegregational killing system based on the noncatalytic *hok-sok* plasmid addiction system from the antibiotic resistance factor pR1. At least one naturally occurring plasmid partition function was also introduced into these expression plasmids, to potentially eliminate random segregation of such plasmids, thereby enhancing their inheritance and stability.

Although these expression plasmids are ultimately intended to express immunogenic and protective antigens for delivery to the human immune system, GFPuv was selected as a test reporter antigen, because quantitation of mean fluorescence in a population of growing live vectors could be used as a measure of the stability of resident plasmids within the live vector. All expression plasmids carried an identical antigen expression cassette, with a P_{ompC1} allele controlling transcription and with translation optimized by incorporation of a consensus ribosome binding site. Because no catalytic activity is associated with the fluorescence of GFPuv, the level of fluorescence intensity measured by flow cytometry within individual bacteria could be correlated directly with gene dosage and copy num-

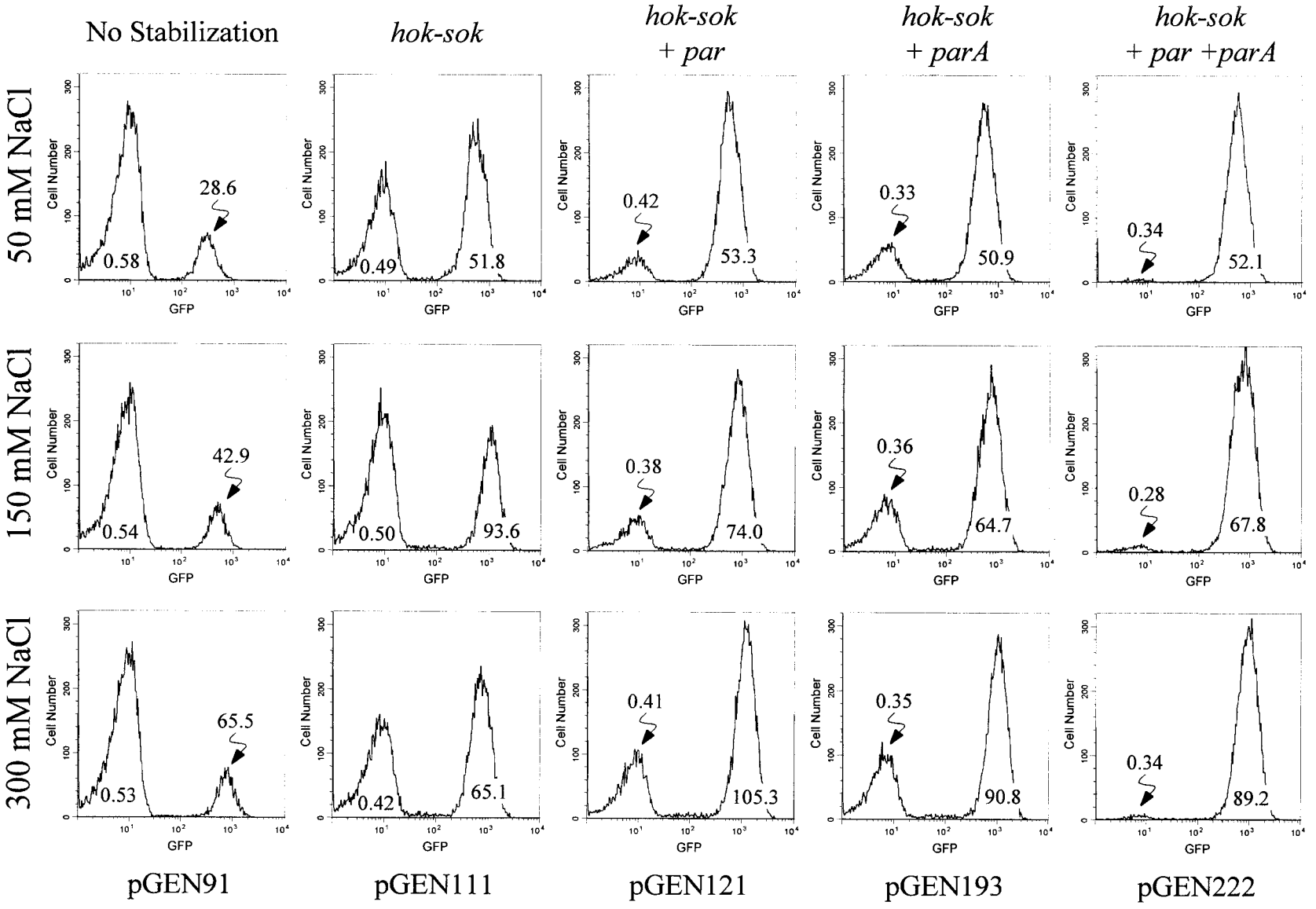

FIG. 2. Flow cytometry histograms of GFPuv fluorescence for populations of attenuated *S. typhi* CVD 908-*htrA* containing expression plasmids with the *ori*15A origin of replication. Histograms are arranged in rows indicating analysis of bacterial populations grown under inducing conditions with either 50, 150, or 300 mM NaCl. The histogram columns indicate CVD 908-*htrA* carrying the expression plasmid listed at the bottom of the column, which contains the maintenance function(s) listed at the top of the column. Results are plotted as the log of GFPuv relative fluorescence intensity versus the number of fluorescing bacteria. The mean fluorescence intensity for a given subpopulation is indicated under each curve; these data are fully quantitated in Table 4.

ber. In addition, use of an osmotically regulated *ompC* promoter allowed an assessment of plasmid stability and live-vector viability as increasing osmolarity induced higher levels of GFPuv synthesis and presumably higher levels of metabolic stress on the live vector. It was surprising and encouraging that although the P_{ompC1} allele was engineered from the chromosomal locus of *E. coli*, it appeared to function more efficiently in *S. typhi* (Table 3).

The contributions of several plasmid maintenance functions to the stability of plasmids within CVD 908-*htrA*, growing in the absence of antibiotic selection, were examined. No combination of maintenance functions could stabilize plasmids containing *ori*E1 origins of replication; in fact, constructs containing maintenance functions were difficult to propagate even in the presence of antibiotic. These observations cast doubt upon the rationale for using higher-copy-number plasmids to optimize expression of heterologous antigens within the cytoplasm of *S. typhi*-based live vectors, a strategy that heretofore has been followed by other groups investigating salmonellae as live vectors (12).

Incorporation of plasmid maintenance functions into plasmids carrying an *ori*15A origin of replication was more encouraging. When live vectors carrying such plasmids were passaged without selection for 24 h at 37°C, the effects of various combinations of maintenance functions became apparent. In the absence of maintenance functions, the *ori*15A replicon pGEN91 was lost from greater than 85% of the population, regardless of the level of induction of P_{ompC1} (Table 4 and Fig. 2). With incorporation of the *hok-sok* postsegregational killing locus in pGEN111, the percentage of bacteria expressing GFPuv tripled under all induction conditions, confirming the observations of others that the *hok-sok* locus enhances the stability of *ori*15A replicons (18, 20, 21). However, it was still noted that regardless of induction conditions, greater than 50% of the bacterial population no longer fluoresced. Since it was confirmed that at least a portion of this nonfluorescing population was still viable and lacked drug resistance, these data confirm previous reports (22, 39, 53) that the presence of a *hok-sok* postsegregational killing system is insufficient by itself to ensure that plasmidless viable bacteria will not arise in a growing population.

One possible mechanism that allows for escape from the influence of *hok-sok* involves spontaneous point mutations arising within the lethal Hok ORF, which could conformationally inactivate Hok and thereby allow plasmid loss to occur without lethality. This point emphasizes the requirement of multiple mechanisms for enhancing the stability of resident plasmids within growing bacteria; should one maintenance function become inactivated, the probability of other independent functions simultaneously becoming inactivated becomes vanishingly small. Indeed, such redundancy in maintenance functions is widespread within naturally occurring low-copy-number plasmids (36). For example, the *E. coli* sex factor F contains one active partitioning function (*sop*) and two killing systems (*ccd* and *flm*) (23, 29, 35, 51). Similarly, the drug resistance plasmid pR1 contains the active partitioning function *parA*, as well as the postsegregational killing system *hok-sok*; in addition, it carries yet another recently defined *kis-kid* killing system (7, 8, 41). We have demonstrated in the work reported here that insertion into multicopy *ori*15A replicons of a more complete maintenance system, composed of both a postsegregational system and two partition functions, dramatically improves the stability of these expression plasmids in the absence of selection, regardless of induction conditions for heterologous antigen expression. However, after passage without selection for 48 h, plasmids were eventually lost from the bacterial population, possibly due to escape from the lethality of Hok. This problem has recently been addressed by Pecota et al. (39), who reported that incorporation of dual killing systems significantly improved plasmid stability compared to the use of *hok-sok* alone; no partition functions were present in these plasmids. Perhaps inclusion of the *kis-kid* killing system, to more fully represent the complement of pR1 stability functions, may be required for optimal stability of higher-copy expression plasmids within *S. typhi* live vectors.

The efficiency of eliciting an immune response directed against a heterologous antigen will depend in part upon the ability of the live vector to present such antigens to the immune system. The ability of a live vector to present antigens will in turn depend upon the stability of multicopy expression plasmids that encode the heterologous antigens. However, we hypothesize that a significant metabolic burden is placed upon CVD 908-*htrA* carrying a multicopy expression plasmid; as copy number and/or level of gene expression increases, metabolic burden increases. Studies with *E. coli* have clearly established that plasmid-bearing bacteria grow more slowly than plasmidless bacteria (6, 30, 39, 46, 53). It has also been demonstrated that as copy number increases, the growth rate of such strains decreases; similarly, as induction of heterologous genes increases, growth rate decreases further (39, 53). Clearly, spontaneous plasmid loss would remove any metabolic burden and allow plasmidless bacteria to quickly outgrow the population of plasmid-bearing bacteria. Such a shift in antigen expression within a population of live-vector bacteria would be expected to reduce the efficiency of stimulating any immune response specific to the foreign antigen. Such reasoning suggests that the goal for an effective multivalent *S. typhi*-based live vector vaccine is to optimize viability by using stabilized lower-copy-number expression plasmids, capable of expressing high levels of heterologous antigen in response to an environmental signal likely to be encountered in vivo after the vaccine organisms have reached an appropriate ecological niche. We are currently testing this strategy by using the murine intranasal model to examine the immunogenicity of protein fusions involving fragment C of tetanus toxin fused at the carboxyl terminus to antigens from the malaria agent *Plasmodium falciparum*, expressed within CVD 908-*htrA* by using expression plasmids derived from pGEN222. It is hoped that such experiments will point the way toward development of single-dose, oral *S. typhi*-based live-vector vaccines capable of inducing protective immune responses against multiple unrelated human pathogens.

ACKNOWLEDGMENTS

This research was supported by grants 5 RO1 AI29471, RO1 AI40297, and Research contract NO1 AI45251 (M. M. Levine, principal investigator).

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Editor: D. L. Burns