APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2000, p. 1216–1219 Vol. 66, No. 3
0099-2240/00/$04.00+0

Development of a Nonantibiotic Dominant Marker for Positively Selecting Expression Plasmids in Multivalent *Salmonella* Vaccines

HESTA V. MCNEILL, KATHARINE A. SINHA,† CARLOS E. HORMAECHE, JEONG JIN LEE, AND C. M. ANJAM KHAN*

Department of Microbiology & Immunology, The Medical School, University of Newcastle, Newcastle NE2 4HH, United Kingdom

Received 20 April 1999/Accepted 10 December 1999

We report the novel application of a herbicide-resistance-based dominant marker for the positive selection of expression plasmids in *Salmonella* serovar vaccines. The β-lactamase gene of the plasmid pTETnir15, which expresses fragment C of tetanus toxin (TetC), has been replaced with the *bar* gene marker. The new plasmid pBAT1 can be positively selected in vitro within *Salmonella* serovars in the presence of the herbicide DL-phosphinothricin. The expression of TetC remains unaltered, and the *Salmonella enterica* serovar Typhimurium vaccine strain is stable and immunogenic in vivo.

The new generation of *Salmonella enterica* serovar Typhi vaccine strains is currently undergoing clinical trials as human typhoid vaccines (18). Such vaccines may also provide potent immunogenic vehicles for the delivery of protective recombinant antigens from other pathogens (8). Multivalent vaccines can be engineered to express cloned antigens from the chromosome as single-copy genes (2, 7). However, a major drawback is that the expression levels of such antigens are often low, resulting in poor immune responses (5). This problem may be circumvented by the expression of antigens from multicopy plasmids, which leads to much-higher expression levels, resulting in stronger immune responses (3).

However, expression plasmids carry antibiotic resistance genes as dominant markers to select for the transformation and inheritance of the plasmid in vitro. As antibiotics are of major clinical importance in the treatment of bacterial infections in humans, a major fear is that resistance genes may spread to pathogenic organisms in the environment. This may render the antibiotic therapeutically ineffective in the treatment of infections with these pathogens. Hence, antibiotic-resistance-based plasmid selection systems are unacceptable for use in humans.

To circumvent these problems, a number of strategies have been used. One approach has been to delete undesirable selective markers from the chromosome by using a site-specific recombination event (11). Alternatively, to stabilize the retention of expression plasmids in vivo, a balanced lethal selection system has been developed (15). A gene conferring resistance to mercury has been successfully used as a selective marker in the development of a live oral cholera vaccine (13). This gene has been used in *Salmonella* serovars; however, growth of the resulting strain became impaired, producing an ineffective vaccine strain (S. Chatfield, personal communication). We have attempted to select plasmids in *Salmonella* serovars by using the luciferase gene, and we have detected transformants by fluorescence (our unpublished observations). However, this too resulted in impaired growth of the transformed *Salmonella* serovars. Thus, a major challenge in the development of live multivalent bacterial vaccines is the identification of dominant markers. Such markers must confer a positively selectable trait, not impair the physiology or immunogenicity of the vaccine strain, and must be clinically acceptable for use in humans.

We report here the novel application of a herbicide-based positive selection system for expression plasmids in live *Salmonella* serovar vaccines. The dominant selective marker is a gene designated *bar* which confers resistance to the herbicide DL-phosphinothricin (PPT) (19, 21). PPT is an analogue of glutamate and a strong and specific inhibitor of glutamine synthetase (14). The *bar* gene encodes the enzyme phosphinothrycin acetyltransferase, which inactivates PPT by transferring the acetyl group from acetyl coenzyme A onto the free amino group of PPT (20). In the absence of glutamine from the growth medium, the *bar* gene can be used as a positively selectable marker for the inheritance and maintenance of plasmids. The *bar* gene has been used with great success by plant genetic engineers as a positively selectable marker gene for transformation (20).

The plasmid pTETnir15 expresses tetanus toxin fragment C (TetC) from the anaerobically inducible *nirB* promoter (3). Mice immunized with *Salmonella* serovars expressing TetC from this plasmid are protected against challenge with lethal doses of tetanus toxin. A serovar Typhi vaccine strain harboring this construct is being considered as a human typhoid and tetanus vaccine. However, the pTETnir15 plasmid carries the ampicillin-resistance (Ap^r) gene encoding β-lactamase, which makes the strain unacceptable for use in humans. In this study, we demonstrate the usefulness of the herbicide-based system by replacing the β-lactamase gene of the plasmid pTETnir15 with the *bar* gene.

Salmonellae are sensitive to the bacteriostatic effects of the herbicide PPT. All bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultivated in M9 medium or M9 agar (4) in the presence or absence of ampicillin or PPT (Sigma). The strains C5HtrA and 541Ty were chosen as model strains for serovars Typhimurium and Typhi (9, 13). Cells from overnight cultures were plated at a series of dilutions onto glutamine-free media supplemented with 0, 2.5, 25, and 250 μg of PPT per ml. At 250 μg of PPT per ml, no

* Corresponding author. Mailing address: Department of Microbiology, The Medical School, University of Newcastle, Newcastle upon Tyne NE2 4HH, United Kingdom. Phone: 44-191-222-7066. Fax: 44-191-222-7736. E-mail: anjam.khan@newcastle.ac.uk.

† Present address: Department of Biochemistry, Imperial College, London SW7 2AZ, United Kingdom.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	References or source
Strains		
SL5338	Serovar Typhimurium *galE* r^-m^+	2
SL3261	Serovar Typhimurium SL1344 *aroA*	1, 6
C5HtrA	Serovar Typhimurium C5*htrA*::Tn*phoA*	9
541Ty	Serovar Typhi *aroA*, *his*, *pur*	12
Plasmids		
pSCB1	Plasmid containing *bar* gene, Ap^R	PBI Cambridge
pTETnir15	Directs the expression of 50-kDa nontoxic TetC, Ap^R	4

growth of these strains was detectable (data not shown). Thus, both serovars Typhi and Typhimurium are sensitive to the effects of the herbicide. Furthermore, the minimal doses of PPT required for its use were established.

Replacement of the antibiotic resistance marker in a *Salmonella* serovar expression plasmid with a herbicide resistance marker. The plasmid expressing TetC, pTETnir15, contains the gene encoding β-lactamase which confers resistance to the antibiotic ampicillin (3). The ampicillin resistance gene was removed and replaced with the herbicide resistance gene by the strategy outlined in Fig. 1 (17). A *bar* gene expression cassette was synthesized by PCR (16) from pSCB1. The PCR was performed by using sense and antisense primers designed to amplify the complete open reading frame of the *bar* gene (5′-TATGAATCAGTTCCATCTACCATGAGCCCAGAACGA-3′ and 5′-TATCTGCAGTTAGATCTCGGTGACGGGCA-3′). This approach had distinct advantages. It allowed the expression of the *bar* gene from the natural ampicillin resistance promoter, while retaining the integrity of the ribosome binding sequence for efficient expression and also permitting the *bar* gene to utilize the signal sequence of the ampicillin resistance gene. Furthermore, this strategy simultaneously resulted in the ampicillin resistance gene being partially deleted and insertionally inactivated.

The *bar* gene in pBAT1 confers resistance to PPT in *Salmonella* serovars. The pBAT1 construct was electroporated into

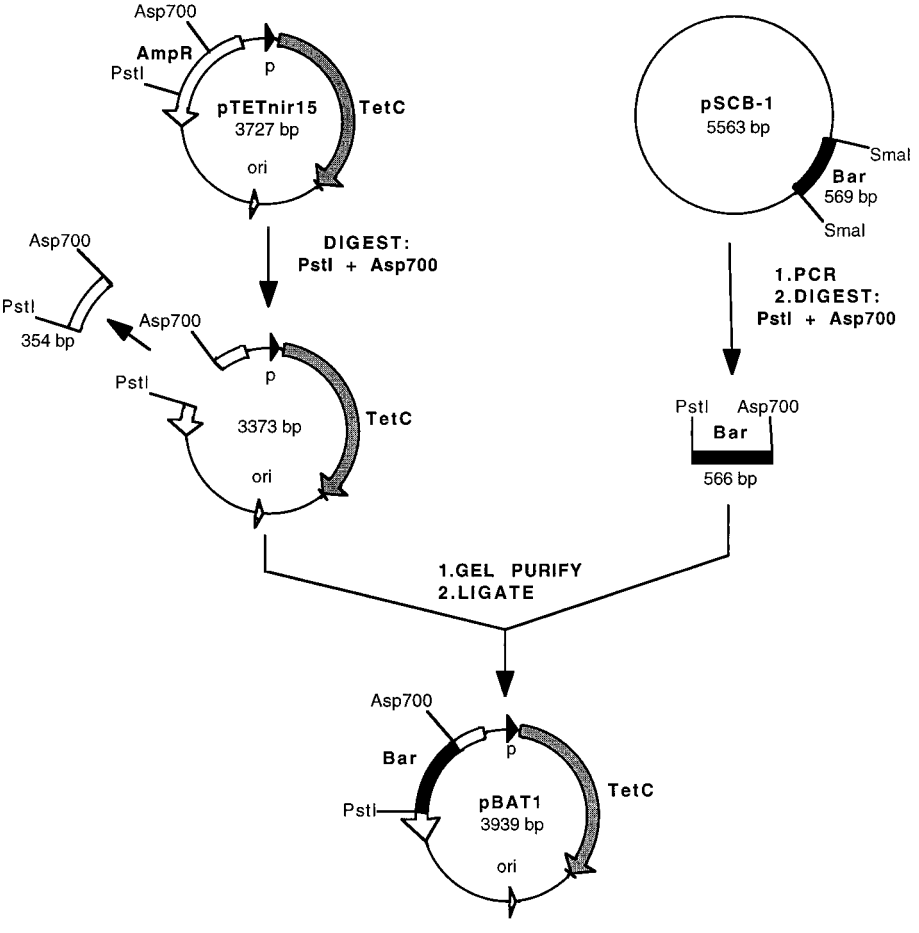

FIG. 1. Strategy for construction of pBAT1.

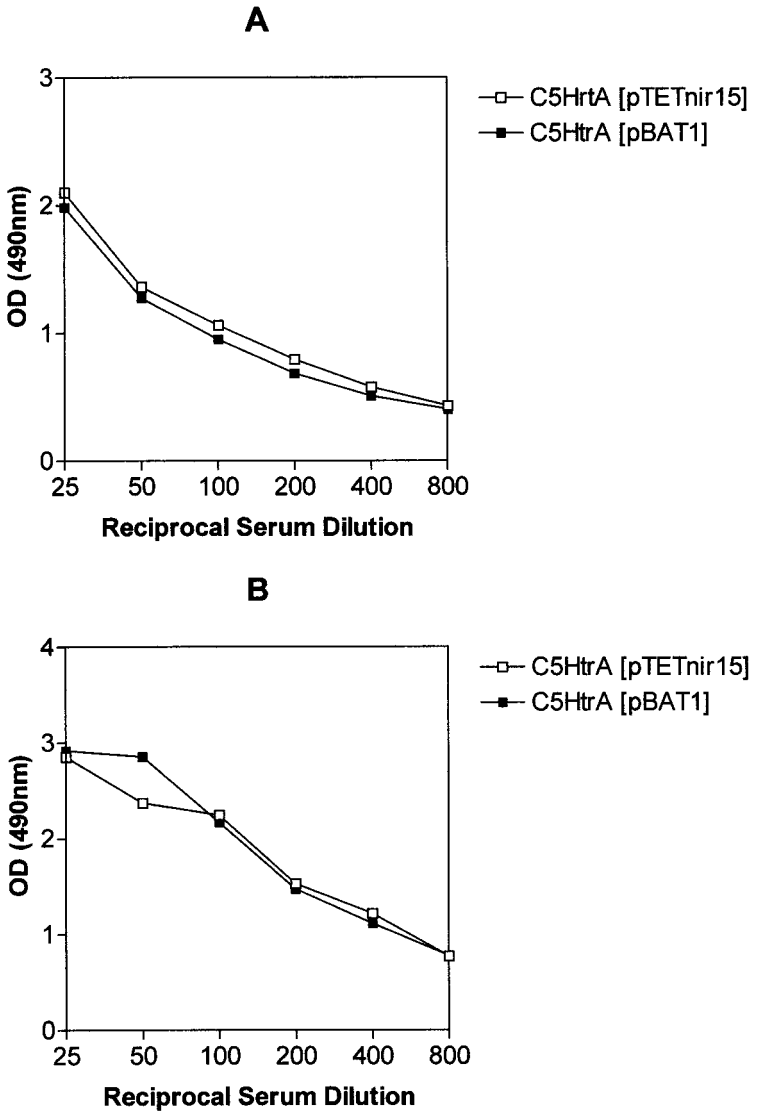

FIG. 2. Immunoglobulin G antibody responses to recombinant TetC as detected by enzyme-linked immunosorbent assay from the pooled sera of mice immunized with C5HtrA(pTETnir15) and C5HtrA(pBAT1). Antibody responses were detected 3 weeks (A) and 5 weeks (B) postimmunization. OD, optical density.

competent C5htrA cells, and transformants were selected in the presence of PPT. Plasmid DNA was purified from transformants, and the identity of the pBAT1 construct was verified by restriction enzyme mapping. Furthermore, the growth of C5htrA(pBAT1) was inhibited in media supplemented with ampicillin, suggesting that there is no remaining residual activity from the partially deleted and insertionally disrupted ampicillin resistance gene as expected. The pBAT1 construct was electroporated into serovar Typhi 541Ty, and transformants were selected in the presence of PPT and verified as above.

Expression of TetC from pBAT1 in *Salmonella* serovars. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were used to compare the ability of C5HtrA(pBAT1) or 541Ty(pBAT1) to express TetC to the ability of the original C5HtrA(pTETnir15) or 541Ty(pTET nir15) strains to express TetC (10). In each case, Western blots were probed with rabbit anti-TetC polyclonal sera, which revealed no visible differences in the expression levels and stability of the 50-kDa TetC band (data not shown). Thus, it can be concluded that there is no aberrant expression of TetC from the pBAT1 construct as compared to pTETnir15 in either of the two *Salmonella* serovars.

Stability in vitro and in vivo of pBAT1 in *Salmonella* serovars. For the *bar* gene to be an effective dominant selective marker of practical value in a vaccine, host vaccine cells should retain the plasmid construct in the absence of marker selection. The ability of pBAT1 to be stably retained in C5HtrA and 541Ty in vitro in the absence of selection was investigated and compared to the ability of pTETnir15 in C5HtrA and 541Ty to be similarly retained.

Strains were grown in liquid media in the presence or absence of the appropriate selective marker. For C5HtrA(pBAT1) and C5HtrA(pTETnir15), the number of colonies counted from liquid cultures which had been grown in the presence and absence of selection yielded the same number of colonies on plates with and without the selective marker (10). The plasmid was segregated and lost from approximately 0.5% of the total

bacterial population per generation (data not shown). This would suggest that the plasmid pBAT1 is as stably inherited as pTETnir15 in the absence of selection with either PPT or ampicillin, respectively.

The in vivo stability of C5HtrA(pBAT1) and C5HtrA (pTETnir15) in intravenously immunized mice was compared at day 10 postimmunization (10). Mice from each group were sacrificed, and the proportion of salmonellae still retaining the constructs and expressing TetC recovered from livers and spleens was determined by Western blotting of 30 randomly picked colonies. The results reveal no differences in the number of salmonellae which retain the plasmid recovered (>90%) (data not shown). Thus, it can be concluded that the *bar* gene product does not place the host cells harboring the construct at a selective disadvantage.

Immunogenicity of pBAT1 in *Salmonella* serovars. The in vivo stability and immunogenicity of C5htrA(pBAT1) was compared to that of C5htrA(pTETnir15). Groups of 10 female BALB/c mice were immunized intravenously with the respective constructs. Sera were taken from mice at weeks 3 and 5 postimmunization. Antibody responses elicited against TetC were measured by enzyme-linked immunosorbent assay as described elsewhere (10). It can be clearly seen in Fig. 2 that both vaccine strains evoked equally strong antibody responses to TetC. This provides convincing evidence that the *bar* gene has no detrimental effect on the immunogenicity of the vaccine strain, and this is, of course, a highly desirable property.

In summary, this study has provided strong evidence to suggest that the herbicide resistance gene *bar* can be used as a dominant marker for positively selecting expression vectors in *Salmonella* serovar vaccine strains. Furthermore, the marker gene fulfills the stringent requirements of not altering the stability or immunogenicity of the recombinant *Salmonella* serovar vaccine strain. Thus, herbicide resistance genes may provide powerful positive selection systems in the development of live multivalent bacterial vaccines which are acceptable for use in humans.

We thank Nigel Batty for valuable discussions.
This work was supported by grants from the Wellcome Trust.

REFERENCES

1. **Brown, A., C. E. Hormaeche, R. Demarco de Hormaeche, G. Dougan, M. Winther, D. Maskell, and B. A. D. Stocker.** 1987. An attenuated *aroA S. typhimurium* vaccine strain elicits humoral and cellular immunity to cloned β-galactosidase in mice. J. Infect. Dis. **155:**86–92.
2. **Chatfield, S. N., N. F. Fairweather, I. Charles, D. Pickard, M. Levine, D. Hone, M. Posada, R. A. Strugnell, and G. Dougan.** 1992. Construction of a genetically defined *Salmonella typhi* Ty2 *aroA aroC* mutant for engineering of a candidate oral typhoid-tetanus vaccine. Infect. Immun. **10:**53–60.
3. **Chatfield, S. N., I. G. Charles, A. J. Makoff, M. D. Oxer, G. Dougan, D. Slater, and N. F. Fairweather.** 1992. Use of the *nirB* promoter to direct the stable expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral vaccine. Bio/Technology **10:** 888–892.
4. **Davis, R. W., D. Botstein, and J. R. Roth.** 1982. A manual for genetic engineering: advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Gonzalez, C., D. Hone, F. R. Noriega, C. O. Tacket, J. R. Davis, G. Losonsky, J. P. Nataro, S. Hoffman, A. Malik, E. Nardin, B. Sztein, D. G. Heppner, T. R. Fouts, A. Isibasi, and M. M. Levine.** 1994. *Salmonella typhi* vaccine strain CVD 908 expressing the circumsporozoite protein of *Plasmodium falciparum*: strain construction and safety and immunogenicity in humans. J. Infect. Dis. **169:**927–931.
6. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–239.
7. **Hone, D., S. Attridge, L. van den Bosch, and J. Hackett.** 1988. A chromosomal integration system for stabilisation of heterologous genes in *Salmonella* based vaccine strains. Microb. Pathol. **5:**407–418.
8. **Hormaeche, C. E., and C. M. A. Khan.** 1996. Recombinant bacteria as vaccine carriers of heterologous antigens, p. 327–359. *In* S. H. E. Kaufmann (ed.), Concepts in vaccine development. Walter de Gruyter, New York, N.Y.
9. **Johnson, K. S., I. G. Charles, G. Dougan, I. A. Miller, D. Pickard, P. O'Goara, G. Costa, T. Ali, and C. E. Hormaeche.** 1991. The role of a stress-response protein in bacterial virulence. Mol. Microbiol. **5:**401–407.
10. **Khan, C. M. A., B. Villarreal-Ramos, R. J. Pierce, G. Riveau, R. Demarco, H. McNeill, T. Ali, N. Fairweather, S. Chatfield, A. Capron, G. Dougan, and C. E. Hormaeche.** 1994. The construction, expression, and immunogenicity of the *Schistosoma mansoni* P28 glutathione *S*-transferase as a genetic fusion to tetanus toxin fragment C in a live Aro attenuated vaccine strain of *Salmonella*. Proc. Natl. Acad. Sci. USA **91:**11261–11265.
11. **Kristensen, C. S., L. Eberl, J. M. Sanchez-Romero, M. Givskov, S. Molin, and V. De Lorenzo.** 1995. Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. J. Bacteriol. **177:**52–58.
12. **Levine, M. M., D. Herrington, J. R. Murphy, J. G. Morris, G. Losonsky, B. Tall, A. A. Lindberg, S. Svenson, S. Baqar, M. F. Edwards, and B. Stocker.** 1987. Safety, infectivity, immunogenicity, and in vivo stability of two attenuated auxotrophic mutant strains of *Salmonella typhi*, 541Ty and 543Ty, as live oral vaccines in humans. J. Clin. Investig. **79:**888–902.
13. **Levine, M. M., D. Herrington, G. Losonsky, B. Tall, J. B. Kaper, J. Ketley, C. O. Tacket, and S. Cryz.** 1988. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet **2:**467–470.
14. **Murakami, T., H. Anzai, S. Imai, H. Satoh, K. Nagaoka, and C. J. Thompson.** 1986. The bialophous biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterisation of the gene cluster. Mol. Gen. Genet. **205:**42–50.
15. **Nakayama, K., S. M. Kelly, and R. Curtiss III.** 1988. Construction of an Asd+ expression-cloning vector: stable maintenance and high level expression of cloned genes in a salmonella vaccine strain. Bio/Technology **6:**693–697.
16. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Ehrlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Tacket, C. O., M. B. Sztein, G. A. Losonsky, S. S. Wasserman, J. P. Nataro, R. Edelman, D. Pickard, G. Dougan, S. Chatfield, and M. M. Levine.** 1997. Safety of live oral *Salmonella typhi* vaccine strains with deletions in *htrA* and *aroC aroD* and immune response in humans. Infect. Immun. **65:**452–456.
19. **Thompson, C. J., N. R. Movva, R. Tizard, R. Crameri, J. E. Davies, M. Lauwerys, and J. Botterman.** 1987. Characterisation of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J. **6:**2519–2523.
20. **Wehrmann, A., A. V. Vliet, C. Opsomer, J. Botterman, and A. Schulz.** 1996. The similarities of the *bar* and *pat* gene products make them equally applicable for plant engineers. Nat. Biotechnol. **14:**1274–1276.
21. **White, J., S.-Y. P. Chang, and M. J. Bibb.** 1990. A cassette containing the *bar* gene of *Streptomyces hygroscopicus*: a selectable marker for plant transformation. Nucleic Acids Res. **18:**1062.