RESEARCH/

USE OF THE *nirB* PROMOTER TO DIRECT THE STABLE EXPRESSION OF HETEROLOGOUS ANTIGENS IN *SALMONELLA* ORAL VACCINE STRAINS: DEVELOPMENT OF A SINGLE-DOSE ORAL TETANUS VACCINE

S.N. Chatfield[1,*], I.G. Charles[2,*], A.J. Makoff[2], M.D. Oxer[2], G. Dougan[3], D. Pickard[1], D. Slater[4] and N.F. Fairweather[2]

[1]Medeva Group Research, Vaccine Research Unit, Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7, UK. [2]Department of Cell Biology, Wellcome Research Labs, Langley Court, Beckenham, Kent BR3 3BS, UK. [3]Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, SW7 2AZ, UK. [4]IDRA Department, Wellcome Research Labs, Langley Court, Beckenham, Kent BR3 3BS, UK. *Corresponding authors.

Plasmid pTET*nir*15, which directs the expression of the non-toxic immunogenic fragment C of tetanus toxin from the anaerobically inducible *nirB* promoter, was introduced into the *Salmonella typhimurium aroA aroD* live oral vaccine strain BRD509. The resulting strain, designated BRD847, was used to vaccinate orally BALB/c mice and was tested for plasmid stability and its ability to protect against a lethal tetanus toxin challenge. pTET*nir*15 was stably inherited by bacteria growing or persisting in the tissues of immunized mice whereas another BRD509 derivative, designated BRD753, harboring plasmid pTET85 which directs fragment C expression from the *tac* promoter, was highly unstable. Mice immunized with a single oral dose of BRD847 developed high levels of circulating anti-fragment C antibodies and were solidly protected against tetanus toxin challenge. Mice immunized with a single oral dose of BRD753 developed no detectable anti-fragment C antibodies. After boosting, antibodies were detected, but the mice were only partially protected against tetanus toxin challenge. Thus the use of an *in vivo* inducible promoter such as *nirB* may be a generally applicable approach to obtaining the stable *in vivo* expression of heterologous antigens in *Salmonella* vaccine strains.

Virulent strains of *Salmonella* can be attenuated by introducing mutations into genes required for their survival and growth *in vivo*. Attenuated strains which establish self-limiting, clinically insignificant infections can be considered as potential live oral vaccines against *Salmonella* infections. For example, Ty21a, an attenuated derivative of *Salmonella typhi* strain Ty2, which harbors mutations in *galE* and other unknown genes, is licensed for use in many countries as a live oral typhoid vaccine[1,2]. Recently genetically defined *Salmonella* strains harboring individual defined mutations have been tested as experimental oral vaccines in several target species. *Salmonella* strains with mutations in genes encoding enzymes of the pre-chorismate metabolic pathway, such as *aroA*, *aroC* or *aroD* which have an auxotrophic requirement for several aromatic compounds, have been shown to be effective oral vaccines in many species including mice[3,4], sheep[5], cattle[6], and chickens[7]. More recently they have been shown to be attenuated and immunogenic in human volunteers[8].

As well as being effective vaccines against salmonellosis, attenuated salmonellae can be considered as vectors for delivering to the immune system heterologous antigens derived from other pathogens. Salmonellae are attractive carriers because they enter the host via the oral route and are potent immunogens being able to stimulate local and systemic cellular and antibody responses. Heterologous antigens from bacteria[9], viruses[10] and parasites[11] can be delivered to the host using this approach.

We have described the construction of a *Salmonella typhimurium* strain expressing the non-toxic, immunogenic 50 kD fragment C derived from tetanus toxin[12]. When used as a live vaccine, this strain was able to protect mice against challenge with tetanus toxin. More recently, we constructed a *S. typhi aroA, aroC* strain, which expresses fragment C from a chromosomally integrated gene[13]. These strains represent the starting point for a combined oral vaccine for humans against both tetanus and typhoid. Mortality from tetanus in the developing world is unacceptably high, with more than one million deaths every year[14]. This is primarily due to problems in the implementation of vaccine programs using conventional toxoid vaccines, and use of an oral vaccine may help to reduce the mortality to tetanus in these countries.

One potentially serious drawback in using live bacterial vaccines for antigen delivery relates to problems with the stability of the foreign antigen expression *in vivo*. Unregulated expression of high levels of a foreign protein in bacteria from multiple-copy plasmids often results in rapid loss of the plasmid or expressed gene from the bacterial cells. This problem can be controlled in fermentors by using inducible promoter systems such as *trp* or *lac*[15] to allow the controlled induction of gene expression when the appropriate biomass has been achieved. These

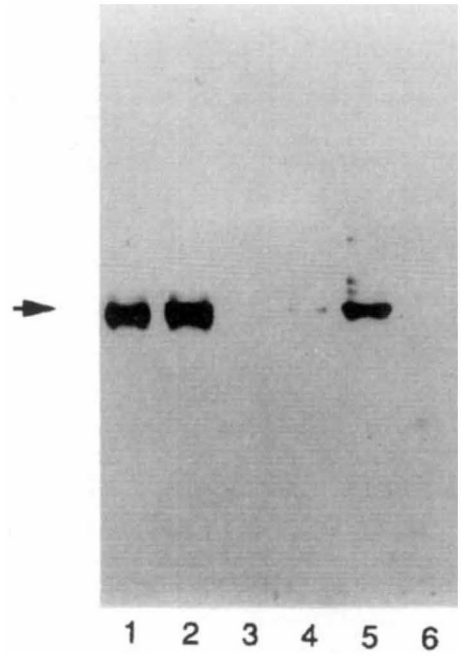

FIGURE 1 Western blot of *Salmonella* strains grown under anaerobic or aerobic conditions. Lanes 1 and 4, BRD847; lanes 2 and 5, BRD753; lanes 3 and 6, BRD509. Lanes 1, 2 and 3 anaerobic growth; lanes 4, 5 and 6, aerobic growth. Fragment C was detected using rabbit anti-fragment C antibody.

promoters require induction by exogenously applied inducers such as IPTG, which is clearly impractical when bacteria are growing in host tissues during the self-limiting growth following vaccination.

A desirable aim for heterologous vaccine development would be to identify a promoter which is tightly repressed under simple fermentor growth conditions but will be induced in particular environments within host tissues. This would ensure appropriate antigen expression in the targeted host tissues without the rapid segregation associated with constitutive expression of foreign proteins. The *nirB* gene, which encodes the *Escherichia coli* NADH-dependent nitrite reductase, is the first gene in an operon that also includes *nirD*, *nirC* and *cysS*[16]. The *nirB* promoter is tightly regulated by nitrite and by changes in the oxygen tension of the environment, and becomes active under anaerobic conditions[16]. We have recently shown that, by altering the oxygen tension in fermentors, the *nirB* promoter region can be effectively used to direct the high level expression of two bacterial antigens; fragment C, the atoxic fragment of tetanus toxin and P.69 pertactin from *Bordetella pertussis*[17]. In this manuscript, we describe the use of the *nirB* promoter to provide the stable expression of fragment C in *S. typhimurium* vaccine strains during self-limiting infection in mice. We demonstrate the utility of this system by showing that these strains can be used as excellent vaccines to immunize mice against tetanus.

RESULTS

Construction of an *S. typhimurium aro* strain expressing fragment C under anaerobic control. pTet*nir*15 is a plasmid which directs the expression of fragment C under *nirB* promoter control[17]. As a step towards constructing a *Salmonella* vaccine strain expressing fragment C under the control of the *nirB* promoter, *S. typhimurium* LB5010 (r^-m^+)[18] was transformed with pTET*nir*15. Ampicillin-resistant (AP-R) transformants of LB5010 which expressed fragment C were detected using colony immunoblotting with anti-fragment C sera. In preparation for immunoscreening, colonies were grown overnight aerobically on nitrocellulose filters and then induced by incubating under anaerobic conditions for four hours. Plasmid DNA was prepared from one fragment isolate which was expressing fragment C, and was used to transform, by electroporation, BRD509 *aroA aroD*[13]. This well-characterised, attenuated *S. typhimurium* strain is an effective single-dose oral vaccine against virulent *S. typhimurium* in BALB/c mice. One transformant that was able to express fragment C stably was chosen for the *in vivo* studies and was designated BRD847.

Fragment C expression in BRD847 was monitored by western blotting cell lysates with anti-fragment C sera after growth under aerobic and anaerobic conditions. Expression levels were compared with BRD753, a BRD509 derivative harboring plasmid pTET85. This plasmid expresses fragment C driven by the *tac* promoter, which in this strain is constitutive because it lacks the *lacI* repressor gene. The results are shown in Figure 1. Expression of fragment C under the control of the *nirB* promoter was considerably induced under anaerobic (lane 1) conditions when compared to aerobic conditions (lane 4). In contrast, when fragment C was under the control of the *tac* promoter, there appeared only a small increase in expression during anaerobiosis (lane 2) when compared to aerobic growth (lane 5). The cause of this apparent induction of the *tac* promoter under anaerobiosis is unclear. However, under aerobic conditions, the level of expression of fragment C under the *tac* promoter is much higher than under the *nirB* promoter (lanes 5 and 4). No expression was seen using the parental strain BRD509 (lanes 3 and 6).

Comparison of plasmid stability and persistence of BRD753 and BRD847 in BALB/c mice. We recently showed that BALB/c mice could be protected against a lethal challenge with tetanus toxin by oral or intravenous (i.v.) immunization with BRD753[12]. Mice required boosting orally with BRD753 in order to be fully protected, possibly because pTET85 was very unstable in bacteria grown in the host and thus fragment C expression was rapidly lost from the immunizing bacterial inoculum. A direct comparison was thus made between the *in vivo* properties of BRD753 and BRD847. Initially a single oral dose of approximately 5×10^9 viable BRD753 or BRD847 bacteria was administered to BALB/c mice and the numbers of viable bacteria which were present in the Peyer's patches, mesenteric lymph nodes, livers and spleens of randomly selected immunized mice were enumerated on selected days after immunization (Fig. 2). Viable counts were monitored on L-agar with or without ampicillin in order to determine the percentage of bacteria which still harbored the recombinant plasmid.

When the bacteria were assayed on L-agar without ampicillin, mice immunized with either BRD753 or BRD847 showed a similar pattern of bacterial tissue colonization and persistence which was consistently lower than that detected for BRD509 (Fig. 2 a–c). However, when ampicillin was incorporated into the agar the levels of colonization by Ap-R BRD753 was much lower than that detected for Ap-R BRD847. The detectable levels of Ap-R BRD753 decreased rapidly following immunization and no Ap-R BRD753 could be detected in the sampled mice tissues by day 14. The levels of Ap-R BRD847 were maintained over the same period. Thus plasmid pTET*nir*15 is much more stable in BRD509 than pTET85 when the strain is growing and persisting in the murine host.

One hundred individual colonies generated from Ap-R BRD847 bacteria passaged *in vivo* were screened for

fragment C expression using colony immunoblotting with anti-fragment C sera. All Ap-R BRD847 colonies tested still expressed fragment C at the levels obtained before immunization.

Serum antibody responses in BALB/c mice immunized with BRD847, BRD753 or BRD509. Groups of twenty mice were inoculated orally with a single dose of 5×10^9 bacteria per mouse of either BRD753, BRD847 or BRD509. Twenty-five days after immunization, sera were collected from all mice and analysed by ELISA for the presence of anti-fragment C antibodies. All mice immunized with BRD847 had detectable anti-fragment C antibody whereas those immunized with BRD753 or BRD509 did not (Fig. 3). On day 25, ten mice from each group were boosted by oral inoculation with a similar amount of homologous organisms. ELISA analysis of the serum taken from these mice at day 46 showed that the anti-fragment C responses had been boosted for groups inoculated with BRD753 and BRD847. The titres for those mice boosted with BRD847 was significantly higher than for those mice boosted with BRD753. Mice boosted orally with BRD509 failed to produce a detectable antibody response to fragment C.

In the same experiment, mice were also given the bacteria intravenously. ELISA analysis of the serum showed that all mice receiving one dose of BRD753 or BRD847 had high titres of anti-fragment C antibodies which were boosted after a second inoculation of the bacteria. The titres of antibodies in the mice receiving BRD847 (containing the pTET*nir*15 plasmid) were consistently higher than those receiving BRD753 (containing the pTET85 plasmid) (data not shown).

Tetanus toxin challenge of mice orally immunized with BRD847, BRD753 or BRD509. The mice vaccinated orally with BRD753, BRD847 and BRD509 were tested for immunity against tetanus toxin challenge after one or two doses of the immunizing strain. Groups of twenty mice received one single oral dose of approximately 5×10^9 organisms and groups of ten mice were challenged on day 25 with 50 LD_{50} of tetanus toxin (see Table 1). Mice vaccinated with BRD847 were completely protected against challenge after a single oral dose whereas those vaccinated with BRD753 were only partially protected (2/10 survivors). The remaining groups of ten mice received a second dose of approximately 5×10^9 organisms on day 25 and were challenged 46 days after the first vaccine dose. Again mice immunized with BRD847 were completely protected against challenge with tetanus toxin whereas those immunized with BRD753 were only partially protected (5/10). Mice immunized with one or two doses of BRD509 all died when subsequently challenged with tetanus toxin. Thus BRD847 can be used as an effective single dose live oral vaccine against tetanus toxin challenge in mice. Groups of mice were also challenged with tetanus toxin after receiving one or two i.v. doses of 10^3 organisms of BRD847 or BRD753. All mice were fully protected against challenge with tetanus toxin after one or two doses of either vaccine strain.

DISCUSSION

We have described the use of the *nirB* promoter to direct stable *in vivo* expression of tetanus toxin fragment C within a *Salmonella* vaccine strain used for heterologous antigen delivery. In contrast to the *tac* promoter, which conferred instability, the *nirB* promoter conferred stability on the expression of the plasmid *in vivo*. *In vivo* plasmid instability during vaccination with live bacterial vectors has been reported by many workers[19,20]. A number of approaches have been taken to overcome the problem including the use of integration systems for expression of the heterologous antigen from the bacterial chromo-

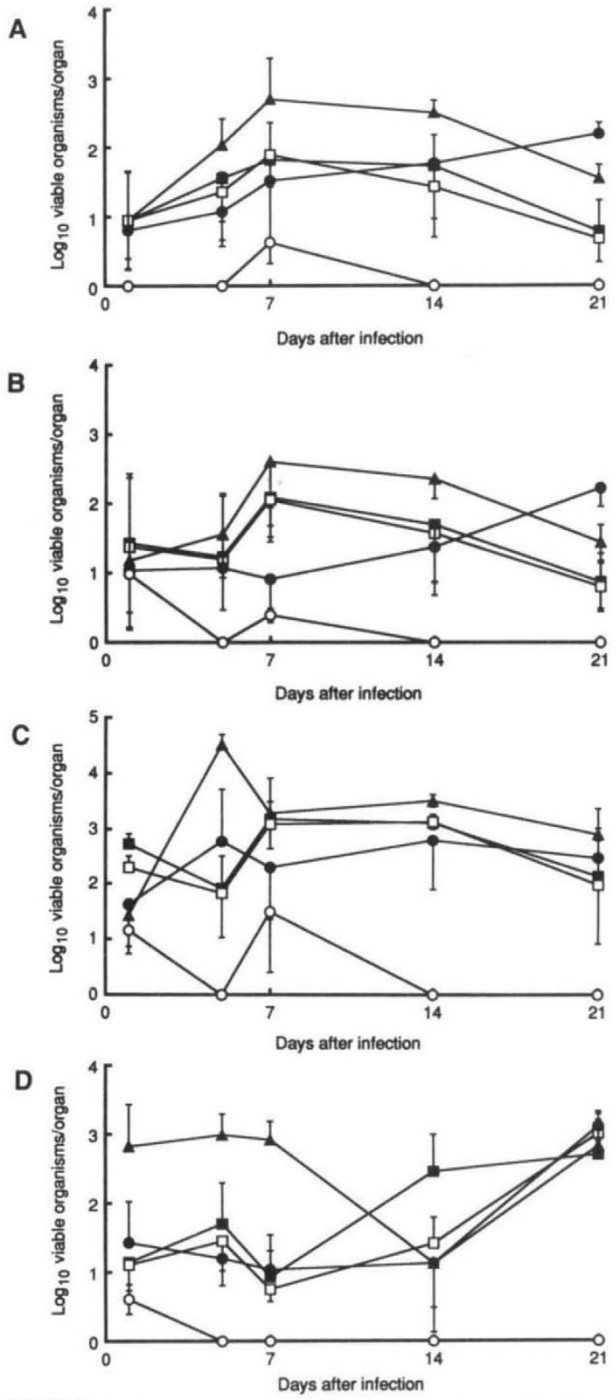

FIGURE 2 Viable counts in (A) livers; (B) spleens, (C) Peyer's patches and (D) mesenteric lymph nodes of BALB/c mice after oral inoculation with bacteria. *S. typhimurium* strain BRD509 (▲); BRD753—ampicillin selection (○); BRD753—no selection (●); BRD847—ampicillin selection (□); BRD847—no selection (■). Data are geometric means ± 2 SE (bars) for four mice per point.

TABLE 1 Oral immunization of mice against tetanus using *S. typhimurium* strains (BRD847, BRD753 and BRD509).

Vaccine	Dose	No. Doses	No. of mice surviving tetanus toxin challenge
BRD509	8.6×10^9	1	0/10
	7.4×10^9	2	0/10
BRD753[a]	6.4×10^9	1	2/10
	8.2×10^9	2	5/10
BRD847[b]	9.5×10^9	1	10/10
	7.5×10^9	2	9/9

Mice were challenged day 25 after 1st dose and day 46 after 2nd dose (given on day 25). [a] BRD753 contains the plasmid pTET85. [b]BRD847 contains the plasmid pTETnir15.

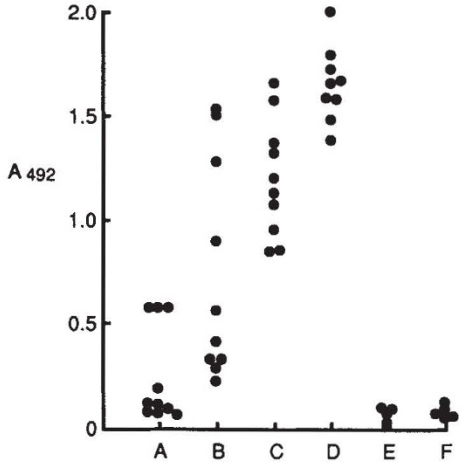

FIGURE 3 Anti-fragment C titres of mouse sera. Sera from individual mice immunized orally with BRD753 (A,B), BRD847 (C,D), and BRD509 (E,F) were prepared as described in the text and 1/500 dilutions were used to react with fragment C coated microtitre plates. A,C,E titres after one dose; B,D,F titres after two doses.

some[19,21]. This approach is not suitable for use with all antigens since expression levels are often quite low[21]. Nakayama et al.[20] described the use of linking an essential gene to the expression plasmid for stabilizing *in vivo* expression. Although this is an effective approach, it does not prevent the generation of plasmid free variants but simply ensures they do not survive. Further, stable but constitutive high level expression of a foreign antigen in a *Salmonella* vaccine strain could slow down the growth rate and hence potentially effect the immunogenicity of the live vaccine. However, this system could be of increased value if it were coupled with an *in vivo* inducible promoter like *nirB*.

The *nirB* promoter is a component of a global regulatory system which responds to both nitrite levels and changes in oxygen tension in the local environment. Response to anaerobiosis is mediated through the FNR global regulatory protein which acts as a general transcriptional regulator of anaerobic respiratory genes[22]. These promoters contain a consensus FNR-binding sequence[23,24] which is included in the constructs described here. At present we do not know in which *in vivo* environments the *nirB* promoter is activated. We found no evidence for plasmid segregation following oral inoculation which suggests expression in the intestinal lumen must be at a level insufficient to cause active segregation, at least with the tetanus fragment C constructs. We are currently using *in situ* immunoblotting to examine the expression levels of fragment C directed by *nirB* in murine tissues and within eucaryotic cells growing *in vitro*. Preliminary experiments indicate that *nirB* may express efficiently once the *Salmonella* have invaded eucaryotic cells (unpublished observations).

Other environmentally regulated promoters may prove useful for expressing heterologous proteins *in vivo*. Work with BCG as a carrier has suggested heat-shock promoters may be of value[25] whereas other applicable promoters could be those sensitive to exogenous metabolites and nutrients such as iron. We have also shown that several other heterologous proteins such as hepatitis B cores (our unpublished results) and schistosome P28 (A. Khan and C. Hormaeche, personal communication) can be stably and efficiently expressed under the control of the *nirB* promoter *in vivo* in *Salmonella* vaccine strains and we are currently investigating the properties of these constructs in mice.

Using the inducible *nirB* promoter to drive fragment C expression, we were able to immunize mice against the lethal effects of a tetanus toxin challenge after only one oral dose of *S. typhimurium* BRD847. This is a considerable advance over our previous work using the *tac* promoter to express fragment C which required two oral doses of vaccine to provide solid protection[12]. When the fragment C gene was introduced into the chromosome of an attenuated *S. typhimurium* strain, we observed stability of the gene, but this was accompanied by a large drop in the expression level[21]. A similar low level of expression of fragment C was seen when the gene was inserted into the chromosome of an attenuated *S. typhi* strain[13]. An attenuated *S. typhi* strain carrying fragment C expressed from the *nirB* promoter using a plasmid based system could be a solution to plasmid instability and would allow high level expression of this antigen. Such a strain would be extremely valuable in developing countries where over one million children die of tetanus every year.

EXPERIMENTAL PROTOCOL

Bacteria, plasmids and growth conditions. *Salmonella typhimurium* LB5010 galE (r^-m^+)[18] and BRD509 (*aroA*$^-$, *aroD*$^-$)[13] have been described previously. Bacteria were routinely cultured on L-agar or in L-broth[26]. Plasmid pTet85 was derived from pTET*tac*115[27] by removal of the 1.2kb EcoRI fragment encoding the *lacI* promoter. pTET*nir*15 has been described previously[17] and encodes fragment C expressed from the *nirB* promoter. Plasmid DNA was introduced into *S. typhimurium* strain BRD509 by electroporation. Bacteria were grown to mid-exponential phase in L-broth, washed once in 10ml cold distilled water containing 10% (v/v) glycerol, and were resuspended to a concentration of 10^{10} cells ml. Sixty μl of cells were mixed with 1μg of DNA resuspended in 5μl of distilled water and the mixture was added to a prechilled 0.2cm cuvette (Biorad, Richmond, CA). The cells were pulsed using a Biorad gene pulser set at 1.75kV, 25uF and 800ohms. This gave a typical pulse time of 12–15 milliseconds. Immediately after pulsing, the cells were added to 1ml aliquots of chilled L-broth, and were incubated at 37°C without shaking for 1.5h. Aliquots were spread over L-agar plates containing ampicillin and were incubated overnight at 37°C.

Infection of mice and enumeration of bacteria in mouse organs. For immunization of mice, bacteria were grown statically overnight in L-broth which contained ampicillin where appropriate, centrifuged and suspended in phosphate buffered saline to approximately 5×10^{10} cells per ml for oral immunization and to approximately 2.5×10^6 for intravenous immunization. Cell suspensions were administered to 6–8 week old male BALB/c mice by gavage tube for oral immunization and by tail injection for intravenous inoculation. Cell viability was determined by counting viable cells in L-agar which contained ampicillin when appropriate. Sera were collected by tail bleeds on the days indicated. Livers and spleens of mice were homogenized as described previously[6] and viable counts were done on these homogenates using L-agar with or without ampicillin as the growth medium.

Western blotting, antibody ELISA and tetanus toxin challenge. Bacterial strains were grown in L-broth containing antibiotics under aerobic or anaerobic conditions, the cells harvested and proteins separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and reacted with anti-fragment C antisera and second antibody as described previously[21]. Sera were analysed for the presence of anti-fragment C antibodies by enzyme-linked immunosorbent assay (ELISA) as described[12] using plates coated with fragment C. The titres were measured as the A_{492} of a 1/500 dilution of the sera. Mice were challenged with 50 LD_{50} doses of tetanus toxin as described previously[12] and survivors were recorded over the next four days.

Acknowledgments

We thank Tina Silva for carefully preparing this manuscript. Received 10 March 1992; Accepted 29 May 1992.

References

1. Germanier, R. and Furer, E. 1975. Isolation and characterisation of *galE* mutant Ty21a of *Salmonella typhi*: a candidate strain for a live oral typhoid vaccine. J. Infect. Dis. **131**:553–558.
2. Levine, M. M., Fericcio, C., Black, R. E., Germanier, R. and Chilean

Typhoid Committee. 1987. Large-scale field trial of Ty21a live oral typhoid vaccine in enteric coated capsule formulation. Lancet **(i):**1049–1052.
3. Hoiseth, S. K. and Stocker, B. A. D. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–240.
4. O'Callaghan, D., Maskell, D., Liew, F. Y., Easmon, C. S. F. and Dougan, G. 1988. Characterisation of aromatic- and purine-dependent *Salmonella typhimurium*: attenuation, persistence, and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56:**419–426.
5. Mukkur, T. K. S., McDowell, G. N., Stocker, B. A. D. and Lascelles, A. K. 1987. Protection against salmonellosis in mice and sheep by immunisation with aromatic-dependent *Salmonella typhimurium*. J. Med. Microbiol. **24:**11–19.
6. Jones, P. W., Dougan, G., Hayward, C., Mackensie, N., Collins, P. and Chatfield, S. N. 1991. Oral vaccination of calves against experimental salmonellosis using a double *aro* mutant of *Salmonella typhimurium*. Vaccine **9:**29–36.
7. Casper, G. L., Nicholas, R. A. J., Cullen, G. A. and Hormache, C. E. 1990. Vaccination of chickens with a *Salmonella enteritidis aroA* live oral *Salmonella* vaccine. Microbiol. Pathogen. **9:**255–265.
8. Dougan, G., Hofnung, M., Lanzavecchia, A. and Leclerc, C. Immune response to proteins with recombinant epitopes, perspectives for vaccines, p. 171–220. *In:*Conferences Philipe Laudat 1990 INSERM, Paris.
9. Newton, S. M. C., Jacob, C. O. and Stocker, B. A. D. 1989. Immune response to cholera toxin epitope inserted in *Salmonella* flagellin. Science **244:**70–72.
10. Tite, J. P., Gao, X-M., Hughes, Jenkins, M., Lipscombe, M., O'Callaghan, D., Dougan, G. and Liew, F. Y. 1990. Anti-viral immunity induced by recombinant nucleoprotein of Influenza A virus. III. Delivery of recombinant nucleoprotein to the immune system using attenuated *Salmonella typhimurium* as a liver carrier. Immunology **70:**540–547.
11. Sadoff, J. C., Ballou, W. R., Baron, L. S., Majarian, W. R., Brey, R. N., Hockmeyer, W. T., Young, J. F., Cryz, J. J., Ou, J., Lowell, G. H. and Chulay, J. D. 1988. *Oral Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. Science **240:**236–238.
12. Fairweather, N. F., Chatfield, S. N., Makoff, A. J., Strugnell, R. A., Bester, J., Maskell, D. J. and Dougan, G. 1990. Oral vaccination of mice against tetanus by use of a live attenuated *Salmonella* carrier. Infect. Immun. **58:**1323–1329.
13. Chatfield, S. N., Fairweather, N. F., Charles, I., Pickard, D., Levine, M., Hone, D., Posada, M., Strugnell, R. A. and Dougan, G. 1992. Construction of a genetically defined *Salmonella typhi* Ty2 *aroA, aroC* mutant for the engineering of a candidate oral typhoid-tetanus vaccine. Infect. Immun: **10:**53–60.
14. Stanfield, J. P. and Galazka, A. 1984. Neonatal tetanus in the third world today. Bull. WHO **62:**647–669.
15. Amann, E., Brosius, J. and Ptashne, M. 1983. Vectors bearing a hybrid *trp-lac* promoter useful for regulated expression of cloned genes in *Escherichia coli*. Gene **25:**167–178.
16. Peakman, T., Crouzet, J., Jayaux, J. F., Busby, S., Mohan, S., Harborne, N., Wooton, J., Nicholson, R. and Cole, J. 1990. Nucleotide sequence, organisation and structural analysis of the products of genes in the *nirB-cysB* region of the *Escherichia coli* K-12 chromosome. Eur. J. Biochem. **191:** 315–323.
17. Oxer, M. D., Bently, C. M., Doyle, J. G., Peakman, T. C., Charles, I. G. and Makoff, A. J. 1991. High level heterologous expression in *E. coli* using the anaerobically-activated *nirB* promoter. Nucl. Acids Res. **19:**1889–1892.
18. Bullas, L. R. and Ryu, J-I. 1983. *Salmonella typhimurium* LT2 strains which are r-m+ for all three chromosomally located systems of DNA restriction and modification. J. Bacteriol. **156:**471–474.
19. Hone, D., Attridge, S., Van den Bosch, L. and Hackett, J. 1988. A chromosomal integration system for stabilisation of heterologous genes in *Salmonella* based vaccine strains. Microbial Pathogen. **5:**407–418.
20. Nakayama, K., Kelly, S. M. and Curtiss, R. 1988. Construction of an *Asd* expression-cloning vector: stable maintenance and high levels expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6:**693–697.
21. Strugnell, R. A., Maskell, D., Fairweather, N., Pickard, D., Cockayne, A., Penn, C. and Dougan, G. 1990. Stable expression of foreign antigens from the chromosome of *Salmonella typhimurium* vaccine strains. Gene **88:**57–63.
22. Jayaraman, P. S., Peakman, T. C., Busby, S. J. W., Quincey, R. V. and Cole, J. A. 1987. Location and sequence of the promoter of the gene for the NADH-dependent nitrite reductase of *Escherichia coli* and its regulation by oxygen, the Fnr protein and nitrite. J. Mol. Biol. **196:**781–788.
23. Bell, A. I., Gaston, K. L., Cole, J. A. and Busby, S. J. W. 1989. Cloning of binding sequences for the *Escherichia coli* transcription activators, FNR and CRP: location of bases involved in discrimination between FNR and CRP. Nucl. Acids Res. **17:**3865–3874.
24. Jayaraman, P. S., Cole, J. A. and Busby, S. J. W. 1989. Mutational analysis of the nucleotide sequence at the FNR-dependent *nirB* promoter in *Escherichia coli*. Nucl. Acids Res. **17:**135–145.
25. Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennet, L. T., Bansal, G. P., Young, J. F., Lee, M. H., Hatful, G. F., Snapper, S. B., Barletta, R. G., Jacobs, W. R. and Bloom, B. R. 1991. New use of BCG for recombinant vaccines. Nature **351:**456–460.
26. Davis, R. W., Bostein, D. and Roth, J. R. 1980. Advanced Bacterial Genetics. A Manual for Genetic Engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
27. Makoff, A. J., Oxer, M. D., Romanos, M. A., Fairweather, N. F. and Ballantine, S. 1989. Expression of tetanus toxin fragment C in *E. coli*: high level expression by removing rare codons. Nucl. Acids Res. **17:**10191–10202.