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# Isolation of stable *aroA* mutants of *Salmonella typhi* Ty2: Properties and preliminary characterisation in mice

**Gordon Dougan[1], Duncan Maskell[1], Derek Pickard[1], and Carlos Hormaeche[2]**

[1] Wellcome Research Laboratories, Langley Court, Wickham Road, Beckenham, Kent, UK
[2] Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

**Summary.** Derivatives of the *Salmonella typhi* strain Ty2 carrying stable mutations in the *aroA* gene were isolated. The mutations were generated by transducing an *aroA*::Tn*10* marker into Ty2 and selecting for derivatives which were tetracyline sensitive and dependent on aromatic compounds for growth. Isolates that did not revert to aromatic compound independence at a detectable frequency were obtained. An *S. typhimurium* derived *aroA* specific DNA probe was used to demonstrate the presence of DNA rearrangements in the *aroA* region of the chromosome of some of the *S. typhi aroA* mutants. Most of these isolates still expressed Vi antigen. Aromatic compound dependent mutants of *S. typhi* were less virulent in mice than *S. typhi* Ty2 following intraperitoneal challenge with bacteria suspended in mucin. Mice immunised with one of these mutants, named WBL85-1, were protected against a potentially lethal challenge of *S. typhi* Ty2.

**Key words:** *Salmonella* – Typhoid – Vaccine – Aromatic

## Introduction

Although the incidence of typhoid fever has been controlled in many countries, the disease remains a serious health problem in some regions of the world (Taylor et al. 1983). Parenteral vaccination with suspensions of dead *Salmonella typhi* has been used for many years and confers significant protection to inhabitants of endemic areas (Ashcroft et al. 1967). Unfortunately, these vaccines regularly cause unpleasant side reaction such as headaches, painful local inflammation and fever; they are also of limited value to subjects with no previous exposure to typhoid (Levine et al. 1983). Since the oral route is the natural portal of entry of *S. typhi* and other enteric pathogens, it is also attractive for the delivery of vaccines against them. Dead whole *S. typhi* vaccines delivered orally were not effective in field trials (Chuttani et al. 1977), whereas attenuated strains of *S. typhi* have shown promise as live oral vaccines. These include streptomycin dependent mutants and *galE* mutants (Reitman 1967; Germanier and Furer 1975). One such strain, known as Ty-21a, contains a stable *galE* mutation and has undergone extensive tests in human volunteers and in controlled field trials (Wahdan et al. 1982).

Certain auxotrophic mutations are known to attenuate the virulence of *Salmonella* strains (Bacon et al. 1951). Hoiseth and Stocker (1981) reported that *S. typhimurium* strains carrying non-reverting deletion mutations in the *aroA* gene were attenuated in mice. This work was based on earlier observations made by Bacon and co-workers using *S. typhi* strains (Bacon et al. 1951). Thus it is feasible that non-reverting *aroA* mutants of *S. typhi* would be attenuated in man and might serve as more effective live typhoid vaccines. In this report we describe the construction of *S. typhi aroA* mutants and their properties in mice.

## Materials and methods

*Bacterial strains, bacteriophage and media. S. typhi* Ty2 was obtained from the Wellcome Research Laboratories culture collection. It derives from the same source as the strain currently employed in the manufacture of whole cell typhoid vaccine. *S. typhimurium* LT2 and LT2 *aroA*554::Tn*10* were obtained from Dr. John Roth, University of Utah, USA. Bacteriophage P22 HT105/1 int$^-$ (Schmieger 1972) was obtained from Tim Foster, Trinity College, Dublin. Bacteria were routinely cultured on L agar plates or in L broth (Davis et al. 1980). Minimal medium consisted of 0.6% $Na_2HPO_4$, 0.3% $KH_2PO_4$, 0.5% NaCl, 0.1% glucose, 0.1% $NH_4Cl$ and 0.025% $MgSO_4$ and was supplemented with nutrients and antibiotics at the appropriate concentrations. Solid media contained 1.2% Noble Agar (Difco, UK). In phage experiments medium was supplemented with 10 mM $CaCl_2$ where appropriate.

*Transduction.* Transduction experiments were carried out using bacteriophage P22 HT105/1 int$^-$ as described elsewhere (Davis et al. 1980). Phage lysates were prepared using host strain LT2 *aroA*554::Tn*10* grown in the presence of 10 mM $CaCl_2$. Transductants were selected on L agar plates containing 50 μg/ml tetracycline and 5 mM ethylene glycol-bis-(beta-amino-ethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) which inhibits phage multiplication. Individual transductants were purified on the same medium until they were free of P22.

*Selection for tetracycline-sensitive mutants.* Tetracycline-sensitive (Tc$^s$) derivatives of Tn*10* were selected using Bochner medium made up exactly as described except that fusaric acid was used at 4 mg/l (Bochner et al. 1980). Bochner medium inhibits the growth of tetracycline-resistant (Tc$^r$) but not Tc$^s$ cells.

*Mouse experiments.* All mice were 8- to 10-week-old male BALB/c bred in the Wellcome Research Laboratory animal house. The lethal dose for 50% of a group of mice ($LD_{50}$) for virulent and attenuated *S. typhi* derivatives was obtained using serial tenfold dilutions, prepared in phosphate-buffered saline pH 7.2 (PBS), of overnight L broth cultures grown at 37° C. A 0.3 ml aliquot of the dilution was mixed with 2.7 ml of hog gastric mucin (Wilson Pharmaceuticals, USA) and 0.5 ml of this preparation was injected intraperitoneally (ip) into each of five mice per dilution. Deaths were recorded over the following week and the $LD_{50}$ was calculated using the method of Reed and Muench (1938). Mice were vaccinated ip using $10^6$ viable *S. typhi* suspended in PBS and challenged ip using different dilutions of viable *S. typhi* prepared from overnight cultures as above, suspended in hog gastric mucin.

*Serology. S. typhi* and *S. typhimurium* strains were routinely tested in slide agglutination tests using appropriate anti-O, anti-H and anti-Vi sera. All sera were provided by Wellcome Diagnostics, UK.

*DNA manipulation techniques.* Whole cell DNA was prepared using the method of Hull et al. (1981). Plasmid DNA was prepared as described in Birnboim and Doly (1981). DNA hybridisations were carried out using the method of Southern (1975). All restriction enzymes and DNA-modifying enzymes were used according to the manufacturer's instructions.

*Isolation of a plasmid encoding the* S. typhimurium aroA *gene.* For safety reasons the *aroA* gene was cloned from *S. typhimurium* C5 (Hormaeche 1979). A cosmid gene bank of C5 was prepared as described previously (Morrissey and Dougan 1986) and introduced into *Escherichia coli* strain AB2829 (Pittard and Wallace 1966). Cosmid recombinants which complemented the *aroA* lesion of AB2829 were isolated by plating onto minimal agar plates. Individual colonies were picked after overnight incubation of these plates, purified and checked for plasmid content. One complementing plasmid was identified and found to contain a 15 kb insert of *S. typhimurium* DNA. The *aroA* determinant was subcloned from this plasmid to yield pAB51 which contains a 3.37 kb fragment of the cosmid vector pHC79 (Hohn and Collins 1980) ligated to a 3.3 kb *Bgl*II-*Sal*I fragment of *S. typhimurium* DNA encoding the *aroA* gene. The restriction map of this fragment closely resembled that previously described for the region of the *S. typhimurium* chromosome encoding the *aroA* gene (Stalker et al. 1985).

## Results

*Construction of non-reverting aroA mutants of* S. typhi *Ty2*

A high titre lysate of phage P22 HT105/1 int$^-$ was prepared using *S. typhimurium* LT2 *aroA*554::Tn*10* as host and dilutions of this lysate were used to infect *S. typhi* Ty2. Tc$^r$ colonies were selected by plating transducing mixtures onto L agar plates containing tetrcycline and EGTA. Individual Tc$^r$ colonies were restreaked on the same medium until they were free of viable P22 HT105/1 int$^-$ phage. Selected colonies were examined for serological properties and their auxotrophic requirements. The parental strain Ty2 was 09 inagglutinable, except after boiling, Vi$^+$ and H-d$^+$. All of the Tc$^r$ colonies examined required tryptophan, tyrosine, phenylalanine, *p*-aminobenzoic acid and 2,3-dihydroxybenzoate for growth in addition to the normal nutrient requirements of Ty2, indicating a lesion in the aromatic pathway. However, serological variants were detected. Some colonies were 09 inagglutinable unless boiled whereas others readily agglutinated in anti-09 antiserum without boiling. Other colonies showed variation in their ability to agglutinate in the presence of anti-Vi antiserum whereas others were fully Vi$^+$. All colonies tested were H-d$^+$.

Several different serologically distinct transductants were grown up and plated onto Bochner medium. After incubation overnight, resultant colonies were tested for tetracycline sensitivity, auxotrophic requirements and by serological methods. Tc$^s$ derivatives were either auxotrophic for the aromatic compounds or grew readily without them, probably indicating imprecise or precise excision of Tn*10*.

A number of aromatic compound dependent Tc$^s$ derivatives were plated at high concentrations onto medium lacking the aromatic compounds to see if *aroA* revertants could be detected. Using conditions that would detect reversion at a frequency of approximately 1 in $10^{11}$, isolates were selected in which no such revertants were identified. Thus the lesion in the *aroA* gene of these *S. typhi* mutants is likely to be a deletion or inversion mutation.

*DNA hybridisation analysis of* S. typhi *Ty2* aroA *mutants using an* aroA *gene probe*

pAB51 is a 6.67 kb recombinant plasmid encoding an *aroA* gene derived from *S. typhimurium* C5 on a 3.3 kb *S. typhimurium* DNA fragment. This plasmid was labelled with $^{32}P$ and used to probe whole cell DNA prepared from *S. typhi* Ty2, Ty2 *aroA*554::Tn*10* and a number of aromatic compound dependent Ty2 derivatives. Figure 1 shows the results of probing the DNA after cleavage with *Cla*I. pAB51 contains a 1.35 kb *Cla*I fragment encoding the *aroA* gene. The probe recognises this fragment in *Cla*I-cleaved DNA prepared from *S. typhimurium* C5 (lane C) and Ty2 (lane E) along with bands of higher molecular weight. The 1.35 kb *Cla*I fragment is absent from the whole cell DNA of *S. typhi* Ty2 *aroA*554::Tn*10* and the aromatic compound dependent Tc$^s$ derivatives of this strain WBL85-1, WBL85-2, and WBL85-3. In the case of Ty2 *aroA*::Tn*10* a new reactive fragment of 3.6 kb is present along with reactive fragments of higher molecular weight. In the case of the aromatic compound dependent, Tc$^s$ derivatives the 3.6 kb fragment is lost. In *Cla*I-cleaved preparations of WBL85-1 DNA two novel bands of 3.2 and 2.75 kb are present. With WBL85-2 and WBL85-3 substantial rearrangements of chromosomal DNA appear to have occurred in the region encoding the *aroA* gene. This was confirmed by probing chromosomal DNA cleaved with other restriction enzymes (results not shown).

*Behaviour of WBL85-1 in mice*

Dilutions of viable WBL85-1 and Ty2 suspended in hog gastric mucin wre injected ip into mice as described in Materials and Methods in order to ascertain the $LD_{50}$ for each strain. Ty2 had an $LD_{50}$ of approximately $10^{3.4}$ whereas the $LD_{50}$ for WBL85-1 was approximately $10^{6.8}$. Clearly WBL85-1 is severely inhibited in its ability to kill mice challenged by this method. Mice were then vaccinated ip with $10^6$ WBL85-1 suspended in PBS. Out of 20 mice vaccinated

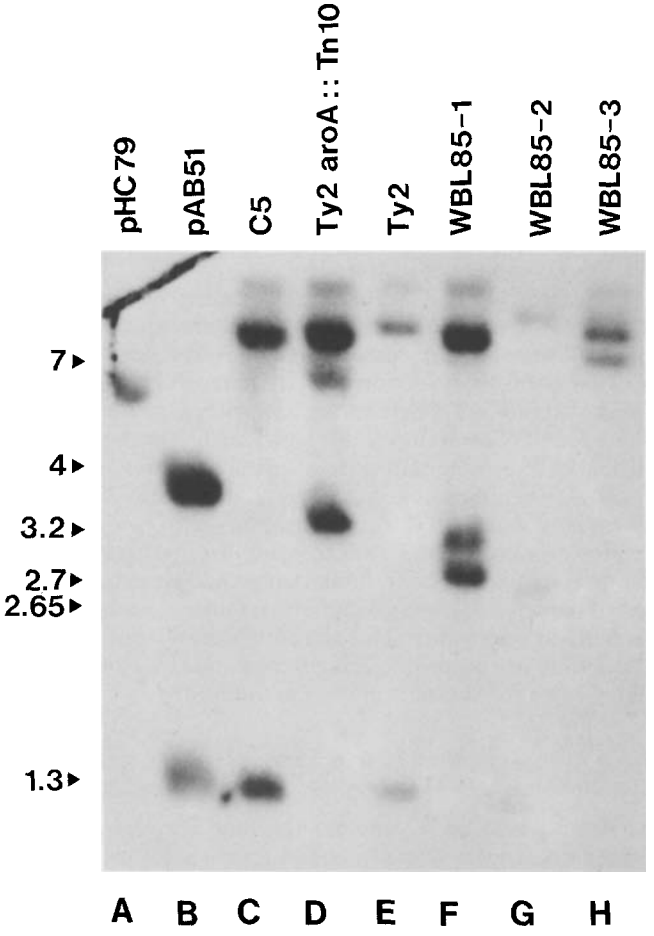


**Fig. 1.** Autoradiograph of Southern hybridisation using $^{32}$P-labelled pAB51 as probe. *Cla*I-cleaved chromosomal DNA was loaded in equal amounts onto a 0.8% agarose gel. Samples applied were: *lane A*, pHC79; *lane B*, pAB51; *lane C*, *Salmonella typhimurium*, C5; *lane D*, Ty2 *aroA*::Tn*10*; *lane E*, Ty2; *lane F*, WBL85-1; *lane G*, WBL85-2; *lane H*, WBL85-3. Molecular weights are as indicated on the left

**Table 1.** Challenge by ip administration in hog gastric mucin by virulent *Salmonella typhi* strain Ty2 to BALB/c mice unimmunised or immunised by ip injection of $10^6$ live *aroA*$^-$ *S. typhi* in PBS

| | Challenge dose | Deaths/no. tested (days to death) | |
|---|---|---|---|
| Unimmunised controls | $1.3 \times 10^6$ | 5/5 (All on day 2) | $Log_{10}$ $LD_{50}$ |
| | $1.3 \times 10^5$ | 5/5 (All on day 2) | $=3.49$ |
| | $1.3 \times 10^4$ | 5/5 (Day 2,2,3,3,3) | |
| | $1.3 \times 10^3$ | 1/5 (Day 2) | |
| Immunised mice | $1.3 \times 10^6$ | 0/5 | $Log_{10}$ $LD_{50}$ |
| | $1.3 \times 10^5$ | 0/5 | $\geq 6.61$ |
| | $1.3 \times 10^4$ | 0/5 | |
| | $1.3 \times 10^3$ | 0/5 | |

none died. Four weeks after vaccination, groups of five vaccinated and normal control mice were challenged ip with different dilutions of viable Ty2 organisms suspended in mucin. The results are shown in Table 1. WBL85-1 was clearly able to protect mice against a lethal challenge of Ty2 organism with an $LD_{50}$ of greater than $10^{6.6}$ in vaccinated mice.

## Discussion

This manuscript describes the construction of a stable *aroA* mutant of *S. typhi* and demonstrates its use as an experimental vaccine in mice. These strains and derivatives of them could find use as live oral typhoid vaccines in humans. Oral administration has proved to be an efficient route for delivery of live vaccines against a number of enteric pathogens including *Vibrio cholerae* and *Shigella* species (Levine et al. 1983). Live vaccines for humans should be attenuated in a well-characterised and stable manner and the mutants generated using the method employed in this paper are of this nature. Bacon et al. (1951) made the important observation that certain auxotrophs of *S. typhi* were avirulent in mice, then Hoiseth and Stocker (1981) employed modern genetic techniques to create stable mutants, using transposon Tn*10*. Imprecise excision of Tn*10* from a site within a gene leads to a high frequency of deletions or inversions of DNA sequences within the gene (Kleckner et al. 1977). *AroA* deletion mutants of *S. typhimurium* and other *Salmonella* species were avirulent in mice challenged via different routes (Hoiseth and Stocker 1981; Maskell et al. 1987). The attenuation is due to the non-availability of aromatic compounds in mammalian tissues. It is still conceivable that a functional *aroA* gene could be restored from another organism, so in practice it might be safer to construct strains of *S. typhi* containing deletions in a number of different *aro* genes. Recently Stocker (1986) has constructed strains of *S. typhi* carrying deletions in *aroA* and *purA*.

WBL85-1 is clearly attenuated in mice compared with the virulent Ty2. Although this result is encouraging it should be interpreted with caution. *S. typhi* does not grow well in mice compared with strains of *S. typhimurium* and it is necessary to challenge mice with *S. typhi* suspended in hog gastric mucin in order to potentiate virulence. The infection caused by *S. typhi* in mice does not resemble typhoid fever but is more like a peritonitis (Carter and Collins 1977). Nevertheless, WBL85-1 does protect mice against Ty2 challenge and this is a test for quality control of killed *S. typhi* vaccines.

*AroA* mutants of *S. typhimurium* protect animals against salmonellosis. Also, live attenuated *Salmonella* vaccines are attracting interest as carriers for delivering heterologous antigens to the secretory and systemic immune systems. The genetic determinants for *E. coli* antigens such as the heat-labile toxin B sub-unit and K88 fimbriae have been expressed in *aroA* mutants of *S. typhimurium* (Kehoe et al. 1981; Dougan et al. 1986; Maskell et al. 1986) and *galE* mutants of *S. typhi* (Clements and El-Morshidy 1984). Intracellular cloned antigens are also immunogenic (Brown et al. 1987), and cloned antigens from *Schistosoma mansoni* have been expressed in *aroA* salmonellae (Taylor et al. 1986). It is feasible that *aroA* mutants of *S. typhi* could find use as multivalent vaccines in humans.

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