Attenuated typhoid vaccine *Salmonella typhi* Ty21a: fingerprinting and quality control

Alison J. McKenna, Jane A. Bygraves, Martin C. J. Maiden
and Ian M. Feavers

Author for correspondence: Ian M. Feavers. Tel: +44 1707 654753. Fax: +44 1707 646730.
e-mail: ifeavers@computing.nibsc.ac.uk

Division of Bacteriology,
National Institute for
Biological Standards and
Control, Blanche Lane,
South Mimms, Potters Bar,
Hertfordshire EN6 3QG, UK

Live attenuated vaccines, developed with molecular genetical techniques, require new approaches for their quality control. To develop novel quality control tests that enhanced and extended existing procedures, the attenuated vaccine strain *Salmonella typhi* Ty21a and its parent strain Ty2 were characterized by pulsed-field gel electrophoresis (PFGE) and direct nucleotide sequence analysis. Mutant and parent strains were distinguished using fingerprints generated by the resolution on PFGE of chromosomal DNA digested with each of the enzymes *SfiI*, *SpeI* or *XbaI*. These fingerprints were stable through multiple *in vitro* passages of the vaccine strain and were identical from one batch of vaccine to another. It was also possible to distinguish between the mutant and parent strains by direct nucleotide sequence analysis of the *galE* gene. This analysis identified two base changes in the gene from strain Ty21a: a single base deletion causing a frameshift that would result in a truncated gene product, accounting for the *galE* phenotype; and a transition that eliminated an *AluI* restriction site. The consequent change in the *AluI* fingerprint of the *galE* gene in strain Ty21a provided a rapid, PCR-based alternative to the use of differential media or biochemical assays for the identification of the vaccine strain.

Keywords: *Salmonella typhi* Ty21a, pulsed-field gel electrophoresis, *galE* gene, nucleotide sequence, live vaccine

INTRODUCTION

For many years inactivated whole cell vaccines have been available for prophylaxis against typhoid fever. These require invasive procedures for immunization and repeated booster doses and are well known for causing unpleasant reactions upon immunization (Ashcroft *et al.*, 1964; Yugoslav Typhoid Commission, 1964; Polish Typhoid Committee, 1966). The discovery that virulent strains of *Salmonella typhi* were attenuated by the introduction of genetic lesions into genes that encode products required for bacterial growth *in vivo* (Germanier & Furer, 1975; Dougan *et al.*, 1987; Chatfield *et al.*, 1989; Hone *et al.*, 1991) led to the development of live, genetically attenuated, typhoid vaccines that established self-limiting immunizing infections. These vaccines had the following advantages: (i) they could be administered

orally; (ii) they did not produce severe reactions in the vaccinee; (iii) they promoted both local and systemic antibody and cellular immune responses; and (iv) they could be used to deliver antigens from other pathogens to the immune system (Clements & El-Morshidy, 1984; Chatfield *et al.*, 1989, 1992; Cardenas & Clements, 1992).

Typhoid vaccine based on *S. typhi* strain Ty21a was the first live attenuated enteric vaccine to be licensed in many industrialized countries, primarily for the immunization of travellers to typhoid endemic areas (Forrest, 1991). The Ty21a strain was derived from the virulent *S. typhi* strain Ty2 by successive rounds of NTG mutagenesis followed by screening for a stable mutation in the *galE* gene, which encodes UDPgalactose 4-epimerase (GalE) (Germanier & Furer, 1975). Attenuation of *Salmonella* strains by *galE* mutations was at that time attributed to their inability to synthesize a complete lipopolysaccharide except under conditions that resulted in galactose-induced lysis (Germanier & Furer, 1983). Strains without a complete lipopolysaccharide were believed to be more susceptible to phagocytosis and therefore less able to survive *in vivo*.

Abbreviation: PFGE, pulsed-field gel electrophoresis.

The EMBL accession number for the sequence reported in this paper is X83927.

During the development of the Ty21a vaccine, no attempt was made to place the *galE* mutation in a strain isogenic with strain Ty2, consequently Ty21a has various mutations in other genes including those affecting: (i) Vi antigen biosynthesis; (ii) isoleucine-valine metabolism; (iii) H₂S production; and (iv) growth rate (Germanier & Furer, 1975, 1983; Hone *et al.*, 1988). Experiments designed to investigate the effect of *galE* alone on the attenuation of *S. typhi* showed that otherwise isogenic *galE* mutants of Ty2 were virulent in human volunteers, demonstrating that *galE* by itself was not the attenuating lesion (Hone *et al.*, 1988).

Strain Ty21a vaccines have proved to be safe (Gilman *et al.*, 1977; Wahdan *et al.*, 1982; Germanier & Furer, 1983; Levine *et al.*, 1987; Black *et al.*, 1990), although their protective efficacy has varied considerably in field trials, probably because of vaccine stability and differences in formulation, vaccination schedule and incidence of infection in the trial population (Hirschel *et al.*, 1985; Forrest, 1991; Forrest *et al.*, 1991; Murphy *et al.*, 1991; Simanjuntak *et al.*, 1991). Strain Ty21a has also been proposed as a vector for the oral delivery of heterologous antigens (Formal *et al.*, 1981; Clements & El-Morshidy, 1984; Chatfield *et al.*, 1989).

The development of quality control tests for vaccines based on Ty21a is hampered by the absence of information on its attenuating mutation(s), the lack of reliable *in vitro* tests and the inadequacy of animal models in predicting the safety and efficacy of typhoid vaccines for man (Germanier & Furer, 1983). Consequently, quality control of these vaccines depends largely upon monitoring the manufacturing process and confirming that the bacterial strain in a given batch is identical to that which was shown to be safe in clinical trials. Molecular genetics provides novel approaches to these problems. The present work describes the application of two molecular techniques to the characterization of the vaccine strain Ty21a and its parent strain Ty2. Pulsed-field gel electrophoresis (PFGE) fingerprinting (Schwartz & Cantor, 1984; Carle *et al.*, 1986; Bohm & Karch, 1992), exploiting restriction endonucleases that cut the chromosome infrequently, permitted the rapid and detailed comparative analysis of the chromosomes of the parent and mutant strains. This approach had potential for confirming the identity and genetical stability of *S. typhi* Ty21a in different batches of vaccine preparation. Direct PCR-based nucleotide sequence analysis permitted the comparison of *galE* genes from strains Ty2 and Ty21a with the sequence previously determined for the *Salmonella typhimurium* LT2 *galE* gene (Houng *et al.*, 1990). Direct nucleotide sequence analysis, although rapid, was not suitable for routine testing, so the resulting data were used to devise a simple test to distinguish Ty2 and Ty21a based on the restriction analysis of the PCR-amplified *galE* gene.

METHODS

Bacterial strains and culture conditions. Cultures of the wild-type strain *S. typhi* Ty2, the mutant Ty21a, and batches of vaccine were provided by the Swiss Serum and Vaccine Institute (Berne). The bacterial strains used in this study were cultured in

brain heart infusion (BHI; Oxoid) broth or on BHI agar at 37 °C unless otherwise stated.

PFGE. This was carried out essentially as described elsewhere (Bygraves & Maiden, 1992). The confluent growth from one agar plate of *S. typhi* grown overnight on BHI agar was scraped into 3 ml TE buffer (10 mM Tris/HCl, pH 8.0; 1 mM EDTA). An even suspension of the cells was made which was then mixed with an equal volume of melted 1.0% (w/v) Sea-Plaque agarose (FMC Bioproducts) in TE at 37 °C and directly dispensed into several pre-cooled plastic moulds (0.1 ml capacity; Pharmacia-LKB) and allowed to set on ice. The plugs were removed from the moulds and incubated overnight at 55 °C in SET (1%, w/v, Sarkosyl; 10 mM Tris/HCl, pH 9.5; 0.5 M EDTA) with pronase E (1 mg ml⁻¹; Sigma). The plugs were washed twice at room temperature in SET without pronase E and stored at 4 °C. Immediately prior to use the plugs were washed three times in TE buffer, incubated with preboiled ribonuclease A (1 µg ml⁻¹; Sigma) at 37 °C for 30 min in the appropriate restriction buffer for the endonuclease (New England Biolabs), and washed twice with the same buffer. For restriction endonuclease digestion, plugs containing chromosomal DNA were equilibrated with the appropriate buffer, melted at 65 °C, and digested with between 5 and 20 U enzyme (New England Biolabs) overnight at 37 °C. The reaction was terminated by the addition of stop buffer (20 mM Tris/acetate, pH 7.5; 0.5 mM EDTA; 1 µg Orange-G marker dye ml⁻¹; 1%, w/v, Sea-Plaque agarose) and heating to 65 °C. Samples were either stored at -20 °C or loaded directly onto gels.

Separation of the digested chromosomal DNA samples by PFGE was carried out with a Pharmacia-LKB 'Pulsaphor' apparatus. Electrophoresis was in a 1% (w/v) agarose gel at a constant voltage of 160 V in 0.5 × TBE (5 mM Tris/borate, pH 8.0; 0.5 mM EDTA), giving a starting current of 80 mA: the electrophoresis buffer was maintained at 14 °C throughout the experiment. An interpolated program was used: 5–10 s pulses in 10 h, followed by 10–30 s pulses in 8 h and 30–40 s pulses in 4 h.

PCR, primers and nucleotide sequence analysis. Reaction components were as follows: 50 ng template (bacterial chromosomal DNA, approximately one eighth of an agarose plug, prepared as above) µl⁻¹; 10 mM Tris/HCl, pH 8.0; 50 mM KCl; 1.5 mM MgCl₂; 0.1% (w/v) gelatin; 200 µM each of dATP, dCTP, dGTP and dTTP; the required primers at 2 µM, and 0.5 U *Taq* polymerase (Amplitaq, Perkin Elmer) per 20 µl reaction. The reactions were incubated for 30 cycles in a PHC-2 programmable heatblock (Technique Instruments) for 2 min at 94 °C, 2 min at 60 °C and 3 min at 72 °C. After 30 cycles the reactions were incubated for a further 3 min at 72 °C. The primers used in this study were based on the nucleotide sequence of the *galE* gene of *S. typhimurium* LT2 (Houng *et al.*, 1990) (Table 1). Primers 157 and 158 were used to amplify the entire coding region of the *galE* for nucleotide sequence analysis by the method of Embley (1991). The PCR products were purified from reaction components by precipitation at 37 °C with 20% (w/v) PEG₈₀₀₀ in 2.5 M NaCl, and washing in 80% (v/v) ethanol. The pellets were dried and resuspended in water (25 µl for a 100 µl amplification). 'Cycle sequencing' was done using *Taq* polymerase and termination mixes from the 'Taquence' sequencing kit (United States Biochemical) with an appropriate primer, radiolabelled by T4 polynucleotide kinase with [γ -³²P]ATP. Each sequence was determined at least once on each strand.

Nucleotide sequences of *galE* genes from various enterobacterial species were compared using programs from the PHYLIP package, version 3.51c (1993) distributed via FTP anonymous at

Table 1. Synthetic oligodeoxyribonucleotide primers used in determining the nucleotide sequences of the *galE* genes of strains Ty2 and Ty21a

The location of the primers and the direction of primer extension, relative to the direction of transcription, are indicated with respect to the coding region of the *galE* gene. The letters U and L in the designation indicate whether the primer corresponds to the upper or lower strand sequence, respectively.

Designation	Direction of primer extension	Nucleotide sequence of primer	Location (base position)
97	Forward	5' GGA AGT CAT ACC TGC GTA CAG TTG 3'	37–60
98	Reverse	5' TTA ATC TGG GTA TGC CTG CGG ATG 3'	994–1017
99U	Forward	5' GCG GCC AAC GTT AAA AAC CTT ATT 3'	337–360
99L	Reverse	5' AAT AAG GTT TTT AAC GTT GGC CGC 3'	337–360
100U	Forward	5' ACC GAG GAT GGC ACC GGC GTA CGC 3'	670–693
100L	Reverse	5' GCG TAC GCC GGT GCC ATC CTC GGT 3'	670–693
157	Forward	5' ATT ACA TGT CAC ACT TTT CGC ATC 3'	Upstream
158	Reverse	5' ATC GAT GGG ATT AAA TGG GGT CAT 3'	Downstream
162U	Forward	5' TTA TAA CCG AAA TTC TGC ACG ATC 3'	191–214
162L	Reverse	5' GAT CGT GCA GAA TTT CGG TTA TAA 3'	191–214

evolution.genetics.washington.edu (directory pub/phylip) by the author J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA, USA. Programs DNADIST and DNAPARS were used to determine the genetic distance between genes and to perform parsimony analyses, respectively. Sequences were aligned manually using the program MACAW, version 2.0.0 Win16 (author Greg Schuler) from the National Center for Biotechnology Information, National Library of Medicine, Bethesda, USA.

Restriction digestion and gel electrophoresis of DNA fragments. The PCR products were purified from reaction components by precipitation at 37 °C with PEG₈₀₀₀, as described above, and were digested with restriction endonucleases essentially as described by Maniatis *et al.* (1982) using restriction endonucleases and buffers supplied by New England Biolabs. Samples of DNA were separated on a 4% (w/v) non-denaturing polyacrylamide gel in TBE buffer, pH 8.0, at 50–100 mA.

Gel visualization and fragment size calculations. Following the resolution of restriction fragments by PFGE or electrophoresis in non-denaturing polyacrylamide, the gels were stained with ethidium bromide and the fragments visualized on an ultra-violet light transilluminator. Gels were photographed and the sizes of restriction fragments were calculated using an electronic gel documentation system (SW2000, UV Products). Bacteriophage λ DNA concatemers (New England Biolabs) for PFGE and a 123 bp ladder (Gibco-BRL) for PCR products were used as references for the calculation of restriction fragment sizes in PFGE and non-denaturing PAGE, respectively.

RESULTS

Restriction endonuclease digestion patterns of *S. typhi* genomic DNA

Separate chromosomal DNA samples from *S. typhi* Ty2 and Ty21a were digested with six restriction endonucleases known to cut bacterial chromosomes infrequently (*NheI*, *NotI*, *SfiI*, *SpeI*, *XbaI* and *XhoI*) and

the resulting fragments were resolved into fingerprints by PFGE (data not shown). The *SfiI*, *SpeI* and *XbaI* fingerprints had 22, 17 and 19 bands, respectively, providing sufficient information to distinguish Ty21a from Ty2 without being too complex (Fig. 1). Fingerprints produced using *NheI*, *NotI* and *XhoI* contained too many bands for all the individual fragments to be clearly resolved. Two additional bands (of 137 and 108 kbp) distinguished the *SfiI* fingerprint of strain Ty21a from that of Ty2, whilst the absence of two fragments (of 380 and 106 kbp) from the *SpeI* fingerprint of strain Ty21a similarly differentiated the vaccine and wild-type strains. The *SpeI* fingerprint of strain Ty21a included an additional fragment of more than 460 kbp, which was consistent with the loss of at least one *SpeI* restriction site during the isolation of the vaccine strain. The differences between the *XbaI*-digest patterns of the two strains were more complex but still permitted the discrimination between mutant *S. typhi* Ty21a and the wild-type strain Ty2. The sizes of the restriction fragments obtained by digesting *S. typhi* with *SfiI*, *SpeI* and *XbaI* are given in Table 2. The total size of the *S. typhi* chromosome could not be calculated accurately from these fingerprints, as some restriction fragments were not resolved by the PFGE conditions used in this study.

To determine the extent to which PFGE fingerprints could be used to differentiate the vaccine strain from other bacteria, the restriction pattern of *XbaI*-digested genomic DNA from strain Ty21a was compared with similar patterns obtained using genomic DNA from seven other enterobacterial species (Fig. 2). Each strain produced a characteristic pattern of fragments that distinguished it from *S. typhi* Ty21a. The *XbaI* fingerprint of *S. typhimurium* LT2 was consistent with the published physical map of its genome (Liu & Sanderson, 1992; Liu *et al.*, 1993).

Fig. 1. Comparison of the chromosomes of *S. typhi* Ty2 and the mutant Ty21a by PFGE. PFGE patterns of genomic DNA from *S. typhi* strains Ty2 and Ty21a (lanes 1 and 2, respectively) digested with (a) *SfiI*, (b) *SpeI* and (c) *XbaI* are shown. Differences between PFGE patterns are indicated by arrows.

Table 2. Sizes of restriction fragments from *S. typhi* resolved by PFGE following the digestion of chromosomal DNA with restriction endonucleases *SfiI*, *SpeI* and *XbaI*

The sizes of the fragments resolved under the PFGE conditions (see Methods) employed in this study were calculated by the SW2000 software package (UV Products) and are given in kbp.

<i>SfiI</i>		<i>SpeI</i>		<i>XbaI</i>	
Ty2	Ty21a	Ty2	Ty21a	Ty2	Ty21a
222.1	220.5		475.5	407.1	
195.7	194.3	388.7			383.2
172.5	170.0	378.0	378.0	320.4	322.6
161.8		281.1	277.1	306.1	308.3
159.3	154.9	263.3	261.4		278.9
146.7	144.7	244.6	242.8	263.2	263.8
	137.4	184.3	184.4	248.2	
133.1	131.4	167.6	166.3	211.9	211.9
126.4	126.4	159.3	158.2	208.9	208.9
121.5	119.9	129.7	129.7	205.0	
113.4	112.6	113.4	112.6	186.7	187.9
	108.4	106.7		159.6	
105.0	104.2	90.8	91.7		153.5
98.0	97.0	82.6	83.5	139.9	139.8
93.6	91.7	68.6	69.4	115.7	116.5
79.9	79.0	47.4	47.4	101.5	103.2
73.7	72.9	41.6	41.6	78.7	79.6
62.1	60.5	32.0	32.0	68.9	69.8
56.8	55.4	28.1	28.1	59.2	60.7
45.6	47.4			50.0	51.9
42.7	41.6				49.4
37.5	36.9			35.9	36.9
30.4	30.4			26.5	27.2
2055.7	2117.0	2807.8	2779.7	2786.3	3054.0

The stability of the restriction endonuclease fingerprints obtained was investigated by passing an isolate of *S. typhi* Ty21a ten times (approximately 80 generations) in BHI and comparing the restriction patterns obtained from the genomic DNA of cells at each passage. There were no changes between the PFGE fingerprints. To determine the stability of the restriction patterns between batches of *S. typhi* Ty21a vaccine, genomic DNA was prepared from cells cultured from five different batches of the enteric-coated formulation of the vaccine and restriction fragments were resolved by PFGE. Once again, no differences were observed between the PFGE fingerprints obtained.

Nucleotide sequence analysis of the *galE* gene

Oligodeoxyribonucleotide primers 157 and 158 (Table 1) were used to amplify the coding region of the *galE* genes of the *S. typhi* strains Ty2 and Ty21a, and the nucleotide sequences of these genes were determined completely on both DNA strands (Fig. 3). Alignment of these sequences revealed that the *galE* gene in the Ty21a mutant differed from that of Ty2 in two bases. The first base change was

a thymidine-to-cytosine transition at base position 367, resulting in the substitution of a proline for a serine in the deduced amino acid sequence of GalE and the elimination of one of the two *AluI* restriction sites in the *galE* gene. The second base change was the deletion of a cytosine residue between base positions 441 and 444 that shifted the reading frame bringing an opal stop codon (base position 461) in-frame. As a consequence, the deduced amino acid sequence of GalE from strain Ty21a is truncated at 153 amino acid residues.

Comparison of enterobacterial *galE* genes

Previous work had shown that the restriction maps of the *galE* genes cloned from *S. typhi* Ty2 and *S. typhimurium* LT2 were very similar (Houng *et al.*, 1990). This was confirmed by comparison of the nucleotide sequences determined in the present study with previously published sequences of the *galE* genes from the enterobacterial species *S. typhimurium* LT2 (Houng *et al.*, 1990), *Escherichia*

Fig. 2. PFGE patterns for *S. typhi* Ty21a are distinct from those of other enteric bacteria. PFGE patterns of *Xba*I-digested genomic DNA from enteric bacteria are as follows. Lanes: 1, *S. typhi* Ty21a; 2, *S. typhimurium* LT2; 3, *Salmonella dublin*; 4, *E. coli* K1; 5, *E. coli* K12; 6, *Enterobacter cloacae*; 7, *Klebsiella aerogenes*; 8, *Klebsiella pneumoniae*. The first and third lanes marked S are λ concatemer molecular size markers and the second and fourth lanes marked S contain λ DNA digested with *Hind*III.

coli K12 (Lemaire & Mueller-Hill, 1986) and *Erwinia amylovora* (Metzger *et al.*, 1994). The genetic distances between these *galE* genes, calculated by the program DNADIST using a sequence alignment generated manually using the program MACAW, are shown in Table 3. The genes from *S. typhi* Ty2 and *S. typhimurium* LT2 were the most similar to one another and both were about the same genetic distance from *E. coli* K12. The same relationships were observed when these nucleotide sequences were subjected to a parsimony analysis with the program DNAPARS (data not shown).

The GalE proteins of *S. typhi* and *E. coli* had 338 amino acids, whereas those from *S. typhimurium* and *Erw. amylovora* had 337 residues because of the deletion of different codons near the 3'-terminus of the gene. The calculated M_r of GalE from *S. typhi* Ty2 was 37 110. The *galE* gene of strain Ty2 differed from that of strain LT2 by 17 nucleotides, giving rise to six amino acid changes: (i) a glutamate substituted for an asparagine at amino acid position 88; (ii) a lysine for an arginine at position 92; (iii) a threonine for an alanine at position 144; (iv) a tyrosine for a threonine at position 149; (v) a serine for a proline at

		Primer 97	
	ATGAGAGTATTGGTTACAGGTGGTAGCGGTACATTGGAAGTCATACCTGCGTACAATTG		60
Ty2	M R V L V T G G S G Y I G S H T C V Q L		
Ty21a	M R V L V T G G S G Y I G S H T C V Q L		
	CTGCAAAATGGTCATGACGTCGTCATCCTCGATAACCTCTGCAACAGCAAGCGCAGCGTG		120
Ty2	L Q N G H D V V I L D N L C N S K R S V		
Ty21a	L Q N G H D V V I L D N L C N S K R S V		
	CTGCCCCGTTATTGAACGTCTGGGCGGTAAGCACCCGACCTTTGTGCAAGGCGATATTTCGC		180
Ty2	L P V I E R L G G K H P T F V E G D I R		
Ty21a	L P V I E R L G G K H P T F V E G D I R		
	Primer162		
	AACGAAGCGCTTATAACCGAAATCTGCACGATCACGCGATTGATACCGTGATTCACTTT		240
Ty2	N E A L I T E I L H D H A I D T V I H F		
Ty21a	N E A L I T E I L H D H A I D T V I H F		
	GCCGGCCTGAAAGCCGTCGGCGAATCGGTGCGCAAGCCGCTGGAGTACTATGACAACAAC		300
Ty2	A G L K A V G E S V A K P L E Y Y D N N		
Ty21a	A G L K A V G E S V A K P L E Y Y D N N		
	Primer 99		
	GTCAACGGTACGCTGCGGTTGGTCAGCGCCATGCGTGCGGCCAACGTTAAAAACCTTATT		360
Ty2	V N G T L R L V S A M R A A N V K N L I		
Ty21a	V N G T L R L V S A M R A A N V K N L I		
	AluI		
	C		
	TTTAGCTCCTCTGCCACCGTTTATGGCGATCAGCCCAAATTCCTTACGTCGAAAGTTTT		420
Ty2	F S S S A T V Y G D Q P K I P Y V E S F		
Ty21a	F S P S A T V Y G D Q P K I P Y V E S F		
	Δ		
	CCTACCGGCACGCCGCAAAGCCCCTACGGCAAAGTAAATTGATGGTAGAACAGATCCTC		480
Ty2	P T G T P Q S P Y G K S K L M V E Q I L		
Ty21a	P T G T P Q S P T A K V N End End		
	ACCGATCTGCAAAAAGCCAGCCGGAGTGGAGTATTGCGCTGCTGCGTTATTTCAATCCG		540
Ty2	T D L Q K A Q P E W S I A L L R Y F N P		
	GTCGGCGCGCACCCGTCGGGCGACATGGGAGAAGATCCGCAAGGTATTCCGAATAACCTG		600
Ty2	V G A H P S G D M G E D P Q G I P N N L		
	ATGCCCTATATCGCCCAGGTGCGCGTGGGTGCTGCGGAATCGCTCGCCGTTTTTCGGCAAC		660
Ty2	M P Y I A Q V A V G R R E S L A V F G N		
	Primer 100		
	GATTACCCGACCGAGGATGGCACCGGCGTACGCGATTACATTACGTTATGGACTTAGCC		720
Ty2	D Y P T E D G T G V R D Y I H V M D L A		
	GACGGGCACGTCGTGGCGATGGAAAACTGGCGGACAAATCCGGCGTACATATTTATAAC		780
Ty2	D G H V V A M E K L A D K S G V H I Y N		
	CTCGGCGGGGTGTGCGCAGTAGCGTCCTGGATGTGGTCAACGCCTTTAGTAAAGCCTGC		840
Ty2	L G A G V G S S V L D V V N A F S K A C		
	GGTAAACCCATTAATTACCACTTCGCGCCGCGTCGCGACGGCGATCTCCCGGCGTATTGG		900
Ty2	G K P I N Y H F A P R R D G D L P A Y W		
	AluI		
	GCGGATGCCAGCAAAGCCGATCGCGAGCTGAACTGGCGCGTCACCCGCACGCTTGACGAA		960
Ty2	A D A S K A D R E L N W R V T R T L D E		
	Primer 98		
	ATGGCGCAGGACACTTGGCACTGGCAGTCACGCCATCCGCAGGCATACTCAGATTAA		1017
Ty2	M A Q D T W H W Q S R H P Q A Y S D End		

Table 3. Matrix of the genetic distances between the *galE* genes of enteric bacteria

The genetic distances were calculated using the DNADIST program from the PHYLIP package.

	<i>S. typhi</i> Ty2	<i>S. typhi</i> Ty21a	<i>E. coli</i> K12	<i>S. typhimurium</i> LT2	<i>Erw. amylovora</i>
<i>S. typhi</i> Ty2	0.0000				
<i>S. typhi</i> Ty21a	0.0010	0.0000			
<i>E. coli</i> K12	0.1634	0.1648	0.0000		
<i>S. typhimurium</i> LT2	0.0170	0.0180	0.1753	0.0000	
<i>Erw. amylovora</i>	0.6261	0.6293	0.6150	0.6480	0.0000

position 337; and (vi) an additional arginine inserted at position 291. The GalE proteins of both *Salmonella* species were also similar to the corresponding protein of *E. coli*, with the majority of amino acid mismatches occurring at the carboxy terminus. The GalE sequence of *S. typhimurium* LT2 had three additional amino acid changes (positions 88, 92 and 144) from that of *E. coli* which were not present in *S. typhi* Ty2.

Detection of the mutant *galE* gene

The change in the *AluI* restriction site at position 424 was used as a rapid test to distinguish between the *galE* genes of strains Ty2 and Ty21a (Fig. 4). Digestion of the PCR-amplified *galE* gene from strain Ty2 with *AluI* resulted in three fragments of 562, 388 and 119 bp (Fig. 4, lane 3), whereas the equivalent PCR product from strain Ty21a digested with *AluI* resulted in fragments of 950 and 119 bp (Fig. 4, lane 2).

DISCUSSION

Molecular techniques provide rapid methods for the detailed analysis of bacterial chromosomes and genes. With the advent of vaccines developed by genetical means, quality control tests based on genetical analyses are necessary. In the present study the techniques of PFGE and PCR-based nucleotide sequencing are used to characterize the oral typhoid vaccine strain Ty21a at the level of the whole genome and of the individual gene, respectively.

Genotypic analyses, by the resolution of restriction fragments of genomic DNA upon PFGE, permitted the direct identification of mutations in the Ty21a vaccine strain. The resulting restriction patterns were characteristic for the vaccine strain and provided information on the physical structure of the chromosome. It was possible to compare the fingerprints from different batches of vaccine and seed lots used in vaccine production. Thus,

the generation of genomic restriction patterns by PFGE provided a comparatively simple and reliable means of confirming the identity and genetical stability of an attenuated strain. Since the differences between genomic restriction fragment patterns of strains Ty2 and Ty21a represented only those nucleotide base changes that either created or eliminated particular restriction sites, PFGE identified a small proportion of the mutational changes in the vaccine strain. That differences between restriction patterns were discerned implied that there were a considerable number of mutations in the attenuated strain.

Direct nucleotide sequence analysis identified the mutations generated in the *galE* gene during the isolation of strain Ty21a. These mutations were consistent with NTG mutagenesis which usually induced transitions but occasionally gave rise to small deletions (Drake & Baltz, 1976). Comparison of the nucleotide sequences of the *galE* genes from both *S. typhi* strains Ty2 and Ty21a revealed that there were two mutations in the *galE* gene of strain Ty21a. The *galE* phenotype of strain Ty21a was attributable to the frameshift resulting from the deletion of one of a run of four cytosine residues between base positions 441 and 444 (Fig. 3) and was consistent with the published observation that single base frameshifts frequently occur within monotonous runs of identical bases (Ripley *et al.*, 1986). This mutation resulted in the expression of a truncated UDPgalactose 4-epimerase and explained the reported stability of the *galE* mutation in strain Ty21a (Germanier & Furer, 1975, 1983), since a frameshift would be less amenable to reversion or suppression than mutations caused by single base changes. It was unknown whether the base transition (T → C base position 367; Fig. 3) in the vaccine strain would cause the *galE* phenotype and contribute to its stability. Comparison of the nucleotide sequences of the *galE* genes of the *S. typhi* strains with those of other enterobacterial species confirmed the reported similarity (Houng *et al.*, 1990) between the *galE* genes of *E. coli* and *Salmonella* species (Table 3).

Fig. 3. Nucleotide and deduced amino acid sequences of the *galE* genes of *S. typhi* strains Ty2 and Ty21a. The complete nucleotide sequence of the *galE* gene of strain Ty2 is shown with the base changes found in the corresponding gene of strain Ty21a indicated above (Δ represents the deletion of a single base). The deduced amino acid sequences are shown below the nucleotide sequences. Sequences corresponding to the primers, listed in Table 1, are underlined.

Fig. 4. Digestion of the PCR-amplified *galE* gene with *AluI* distinguishes *S. typhi* Ty2 from strain Ty21a. The *galE* genes of strain Ty21a and Ty2 were amplified by the PCR using primers 157 and 158, digested with *AluI*, and resolved on a 4% non-denaturing polyacrylamide gel. Lanes: 1 and 2, the PCR product of strain Ty21a uncut and cut with *AluI*, respectively; 3 and 4, the PCR product of strain Ty2 cut with *AluI* and uncut, respectively; 5, a 123 bp size marker.

The data obtained in the present study show that molecular genetical techniques such as PFGE and PCR can be applied in the quality control of oral typhoid vaccine and have potential for use in the quality control programmes for other live attenuated bacterial vaccines. These approaches are precise and rapid, as well as being reasonably inexpensive. Existing procedures for the quality control of the *S. typhi* Ty21a vaccine, which are intended to demonstrate whether the strain in a particular vaccine batch is identical to that previously shown to be safe in clinical trials, are based exclusively on the phenotypic characterization of the strain isolated from the

batch of vaccine under examination. Following the chemical mutagenesis of strain Ty2, the *galE* mutation was not reintroduced into Ty2 to ensure that the vaccine strain was isogenic, consequently Ty21a possesses numerous additional mutations many of which are not exhibited as easily detectable phenotypic traits. Currently, identity tests for this vaccine confirm the presence of the mutations that result in easily detectable alterations in the biochemical and antigenic characteristics of the strain, such as the *galE* phenotype, the failure to produce H₂S, or the inability to synthesize the polysaccharide Vi antigen. These characteristics are not necessarily responsible for the attenuation of the strain, their detection depends on phenotypic expression, and they represent only a small proportion of the genome. Consequently, they cannot be used to assess the safety of the strain, they are poor indicators of the identity of the Ty21a strain, and cannot be used to assess its genetical stability.

Although it has been shown in volunteer studies with isogenic *galE* derivatives of strain Ty2 that *galE* is not the mutation responsible for attenuation (Hone *et al.*, 1988), the *galE* phenotype is used as a marker for the identification of strain Ty21a in the current quality control procedures for this vaccine. The base transition at position 427 (Fig. 3) that eliminated an *AluI* restriction site in the vaccine strain provided an alternative, genotypic test for the identification of the vaccine strain. This was conveniently demonstrated by the *AluI* digestion of the PCR-amplified *galE* gene. It had distinct advantages over the existing tests for *galE*, which involve biochemical assays and growth on differential culture media; it was not dependent upon the phenotypic expression of *galE* and, since it was based on PCR, could be performed directly on vaccine samples without requiring further subculture.

The molecular genetical approach described in the present study can be applied to the quality control testing of other live bacterial vaccines. Although the use of BCG vaccine is long established, the vaccine strains are relatively poorly characterized and their identity is assumed from their slow growth and the microscopic examination of acid-fast stained smears. Data from PFGE and PCR-based methods would enhance the existing identification of BCG vaccine strains used by different manufacturers and permit analysis of the genetical stability of the strains between batches. In addition to existing live vaccines, strains of *S. typhi* with mutations in the genes encoding enzymes of the pre-chorismate metabolic pathway (such as *aroA*, *aroC* and *aroD*) and strains of *Vibrio cholerae* lacking the *ctxA* gene (but resistant to Hg⁺⁺) have both been shown to be attenuated and immunogenic in human volunteers (Dougan *et al.*, 1990; Levine & Kaper, 1993; Dougan, 1994). The direct examination of the genome, utilizing both PFGE fingerprinting and PCR-based methods for the detection of their respective attenuating lesions, will expedite the quality control of rationally designed, attenuated bacterial vaccine strains such as these when they become available. Similar methods utilizing PCR have been employed to distinguish the attenuating lesions of viral vaccine strains (Fineschi *et al.*, 1992; Scherba *et al.*, 1992; Mori, 1994; Takeda *et al.*, 1994).

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