

The production of an auxotrophic marked, plasmid-cured *Salmonella* ser. Typhimurium as a live attenuated vaccine

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ABSTRACT

VAN DER WALT, MARTHA L. & GREEFF, A.S. 1998. The production of an auxotrophic marked, plasmid-cured *Salmonella* ser. Typhimurium as a live attenuated vaccine. *Onderstepoort Journal of Veterinary Research*, 65:213–220

A number of amino acid requiring auxotrophic strains of *Salmonella* Typhimurium were produced by chemical mutagenesis. One of them, strain 81, was cured of the virulence plasmid and attenuated for mice. This strain had an auxotrophic requirement for serine, which could be used as a marker for the differentiation of the vaccine strain from other isolates in the field. The strain still contained the smooth form of the O-antigen, was resistant to Complement-mediated killing of serum and produced type 1 fimbriae. Of the six auxotrophic mutants only this mutant differed in its outer membrane protein profile from that of the parent strain in that an outer membrane protein of about 30 kDa was absent. With the use of the polymerase chain reaction, using total DNA of the cell as template, and with primers targeted to the virulence plasmid, it was shown that the virulence plasmid of *Salmonella* Typhimurium was completely cured from this strain. This strain also had a LD₅₀-value of 4 log units lower for mice than the parent strain. The plasmid-cured strain gave a very high degree of protection to mice after systemic immunization, but not after oral vaccination. Compared to the parent, strain 81 also had a lower multiplication rate in the liver and spleen after intraperitoneal inoculation, characteristics that could be attributed to plasmid-loss, and it could also not be recovered from the spleen and liver of orally inoculated mice.

Keywords: Amino acid, auxotrophic, *Salmonella* Typhimurium, strain

INTRODUCTION

In the search for effective vaccines to combat *Salmonella* infection of livestock, world-wide research has focused on the development of live, smooth, attenuated vaccines. The reason for the superior immunity conferred by infection with live organisms is believed to be the result of their persistence in the

host, thus providing sustained antigenic stimulation of the immune system (Collins 1974; Collins & Carter 1974; Muotiala, Hovi & Makela 1989; Hsu 1989). In addition, the smooth O-antigen form of the infecting organisms is required for the stimulation of the humoral response (Murray 1986). The attenuated characteristic must be very stable to reduce the possibility of reversion to virulence. Several methods of attenuation have been investigated. One of the virulence factors of *Salmonella* Typhimurium is a high molecular mass plasmid that has been shown to contribute to its virulence for mice (Gulig 1990). Plasmid-cured strains have been isolated and found to have substantially lower virulence than plasmid-containing strains. Terakado, Sekizaki, Hashimoto & Naitoh (1983) were the first to suggest that such plasmid-cured strains may serve as effective attenuated vaccines. Subsequently, mice were effectively vaccinated with serovar Dublin (Fierer, Chikami, Hatlen,

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Article submitted as partial fulfilment for the D.Phil degree at the Medical University of Southern Africa

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Accepted for publication 3 July 1998—Editor

Heffernan & Guiney 1988), and serovar Enteritidis (Nakamura, Sato, Ohya, Suzuki, Ikeda & Koeda 1985), while chickens were protected after vaccination with a plasmid-cured strain of Gallinarum (Barrow 1990). The results of these experiments showed that plasmid-cured strains were still immunogenic and that these strains, when used as live, attenuated vaccines, held promise. The aim of this study was to develop a smooth, plasmid-cured strain of *Salmonella* Typhimurium and to determine if such a strain would still be immunogenic for mice. Apart from the attenuative characteristic of a live vaccine strain that has to be stable, it is also required that such a vaccine should carry a marker for the differentiation of this vaccine strain from field strains. It was decided to generate auxotrophic mutants from a prototrophic strain. The auxotrophic characteristic could then be used as a marker, independent of the attenuative characteristic.

MATERIALS AND METHODS

Production of auxotrophic mutants

A wild-type, calf-virulent strain of *Salmonella* Typhimurium, strain 5310, was used as the parent strain for the production of the auxotrophic mutants. Auxotrophic mutants were produced by chemical mutagenesis with an acridine half-mustard mutagen and identified by the replica plate technique as described by Carlton & Brown (1981). Six auxotrophic mutants were produced, viz 3a, 6, 17, 81, 102 and 212.

Surface antigen analysis of the auxotrophic mutants

O-antigen analysis

The smooth/rough (S/R) form of the *O*-antigens was determined by examination of extracted lipopolysaccharide (LPS) molecules by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). LPS molecules were isolated by the hot phenol method as described by Westphal & Jann (1965) and separated on a 12% SDS-PAGE as described by Hitchcock & Brown (1983). The molecules were detected in the gel by silver staining. The smooth/rough form of the *O*-antigens was also determined by the resistance of the strains to the bactericidal effect of serum as described by Poppe & Gyles (1987), as well as by agglutination in 0,5% acriflavine saline.

Outer membrane protein (OMP) analysis

Outer membrane proteins were isolated as described by Chart (1994). The proteins were separated on a 12% SDS-PAGE gel and visualized by staining with Coomassie brilliant blue.

Determination of type 1 fimbriae formation

For the optimal production of fimbriae, strains were grown in nutrient broth at 25°C as described by Old & Duguid (1970). The formation of type 1 fimbriae was determined by mannose-sensitive haemagglutination in 3% guinea-pig red blood cells, as described by Jones & Richardson (1981). The production of fimbriae by strain 81 was compared to that of strain 5310.

Plasmid isolation and polymerase chain reaction amplification of the virulence plasmid

Plasmids were isolated according to the method of Birnboim & Doly (1979) and analysed on a 0,7% agarose gel. Plasmids prepared from a strain of *Escherichia coli*, NCTC 50192 (National Collection of Type Cultures, Public Health Laboratory Service, London, United Kingdom), were included as molecular mass markers. Primers for the polymerase chain reaction (PCR) were selected to be complementary to regions of a sequenced gene on the virulence plasmid (Norel, Pisano, Nicoli & Popoff 1989; Gulig & Chiodo 1990). The primer set generates a 456 bp amplified DNA fragment. The sequence of primer 1 (Department of Biochemistry, University of Cape Town, Rondebosch, 7700 South Africa) was CCACTTTAAAGAGGCGCTGGATG (5'→3') and that of primer 2 (Department of Biochemistry, University of Cape Town, Rondebosch, 7700 South Africa) was CCgACTCAGgACACTgTC. Template DNA for the reaction was total cellular DNA released from the cells with Genereleaser (Whitehead Scientific). The concentration of the primers was 200 pMoles per 20 µl of PCR reaction volume, that of the deoxynucleoside triphosphates (Promega) was 200 µM, and 2,5 U *Taq* DNA polymerase (Promega) per reaction was used. A MgCl₂ concentration of 2,5 mM was used. Annealing was done at 45°C for 1 min, denaturation for 1 min at 94°C and extension at 72°C for 1 min, for 30 cycles on a thermal cycler (OmniGene Hybrid). The product of amplification was determined on a 1% agarose gel at 60 V for 2 h. The DNA molecular mass marker, pGEM (Promega), was used.

Determination of attenuation for mice of auxotrophic strains

In all experiments, male Swiss White mice (Laboratory Animal Section, Onderstepoort Biological Products, Onderstepoort, 0110 South Africa), 6 weeks of age, were used. They were of the *Salmonella* Typhimurium resistant line (Robson & Vas 1972).

LD₅₀ virulence determinations

To determine the LD₅₀ value, groups of ten mice were inoculated intraperitoneally with serial dilutions of approximately 5 log 8 colony forming units (cfu)/ml

bacteria in phosphate buffered saline (PBS). The number of animals which survived the infection after 3 weeks was used to calculate the LD₅₀ value according to the method of Reed & Muench (1938).

Determination of the difference in virulence of strain 5310 and strain 81 by oral inoculation and intraperitoneal inoculation of mice

Results from the LD₅₀ determinations revealed that strain 81 had the lowest value of the three auxotrophic mutants that still possessed the smooth form of the O-antigen, and therefore only this mutant and strain 5310 were used for further studies in mice.

Two groups of 36 mice each, were inoculated intraperitoneally with log 5 cfu/mouse of strain 5310 or 81 respectively. At time intervals of 1, 3 and 6 h, and 1, 4, 7, 10, and 14 d and 3, 4 and 5 weeks, three mice from each group were killed. The spleens and livers were homogenized in PBS in a 1/10 ratio (mass/volume). Ten-fold serial dilutions of the homogenates were made in PBS and 0,1 ml of the dilutions were inoculated onto MacConkey's agar (MC agar) and the number of salmonellas per gram organ was determined. In another experiment, two groups of 18 mice each were orally dosed with 0,2 ml of 5 log 7 cfu/ml of either strain 5310 or strain 81. At specific time intervals (1, 3, 6, 24 and 48 h), three mice from each group were killed. Portions of the livers and spleens were directly inoculated onto MC agar and into selenite F broth enriched for *Salmonella*.

Vaccination of mice with strain 81 and challenge by virulent parent strain 5310

Three routes of vaccination, i.e. oral, intraperitoneal and subcutaneous, were studied. The oral and intraperitoneal vaccination doses were log 4 cfu/mouse and the subcutaneous vaccination dose was log 7 cfu/mouse. Groups of 20 mice were injected twice, 1 week apart, with strain 81. Control mice, also 20 per group, were injected with PBS. Two weeks after the second vaccination all mice were challenged intraperitoneally with log 7 cfu of strain 5310/mouse, a dose predetermined to kill more than 50% of unvaccinated mice. Deaths were recorded 2 weeks after challenge and the percentage survival was determined.

RESULTS

Analysis of the auxotrophic mutants

After chemical mutagenesis of strain 5310 a total of 2,5 log 5 colonies was analysed and six auxotrophic mutants were identified (Table 1). The mutagenic treatment did not influence other biochemical characteristics of the mutants (data not shown).

Surface antigen analysis

SDS-PAGE of the O-antigenic preparations of auxotrophic mutants 3a, 212 and 81 and the smooth parent strain 5310 (Fig. 1) showed that they possessed the smooth form of the O-antigen (Palva & Makela

TABLE 1 Amino acid requirements of auxotrophic mutants produced from strain 5310 of *Salmonella* Typhimurium detected by replica plating methods

Mutant strain	Auxotrophic for
17	leu
81	ser
212	his
6	phe, tyr, his, leu
102	his, leu, ile, val, lys
3a	his, phe, gln, leu, tyr, ser, met, val, thr, asn, lys, pro, arg

Abbreviations: arg = arginine
 asn = aspartic acid
 gen = glutamic acid
 his = histidine
 ile = isoleucine
 leu = leucine
 lys = lysine
 met = methionine
 phe = phenylalanine
 pro = proline
 ser = serine
 thr = threonine
 tyr = tyrosine
 val = valine

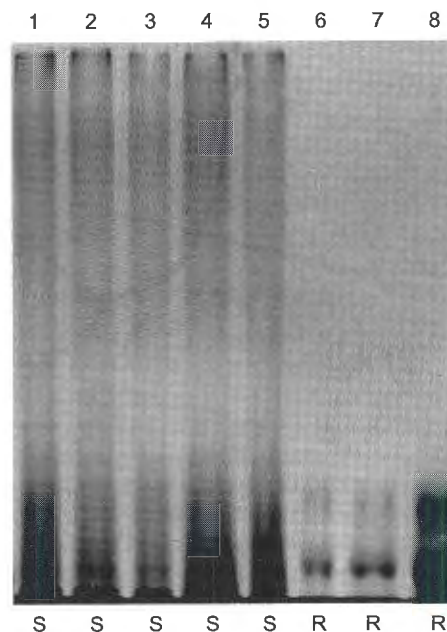


FIG. 1 PAGE of the O-antigens of parent strain 5310 and its auxotrophic mutants strains

Lane 1 = strain 3a
 Lane 2 = 5310
 Lane 3 = 212
 Lane 4 + 5 = 81
 Lane 6 = 6
 Lane 7 = 17
 Lane 8 = 102

S = smooth form of the O-antigen
 R = rough form of the antigen

1980; Hitchcock 1983; Helander 1985). The O-preparations of auxotrophic strains 6, 17 and 102 were typical of the rough phenotype. The results of agglutination in acriflavine saline were in comparison to that of SDS-Page with regard to the S/R character of the O-antigens.

The parent strain 5310 and mutant strain 81, both with the S form of the O-antigen, showed $\geq 100\%$ increase in growth in the complement (C')-positive serum suspension (Table 2). The value for strain 102, a rough strain, was less than 100%. All three strains were able to multiply well in the complement-inactivated serum suspension, an indication that it was the complement part of the serum which inhibited growth of strain 102.

A protein of 30 kDa, present in the parent strain and the other auxotrophic strains (Fig. 2), was absent in strain 81. No other difference in the OMP preparations of the mutants and the parent strain could be observed. The haemagglutination of both strains 5310 and 81 was inhibited by the presence of 0,5% mannose, an indication that both strains produced type 1 fimbriae (data not shown).

Virulence of auxotrophic strains for mice and vaccination of mice with strain 81

The LD₅₀ value of the parent strain was at a value of log 4,27 dilution of 5 log 8 cfu/mouse (Table 3). All the auxotrophic mutants had lower values.

Strain 5310, after oral inoculation into mice, passed rapidly through the intestinal wall and could be detected with enrichment in spleens and livers 60 min post inoculation (Table 4). Thereafter it was present in high enough numbers in the organs for detection by direct inoculation of the organs onto the agar medium. The results obtained with strain 81 were in contrast to those of strain 5310, in that it could not be recovered from the livers and spleens after oral dosing of mice.

In the experiment in which mice were inoculated intraperitoneally with either of the strains followed by subsequent determination of the numbers of bacteria in the intestinal organs over a period of time, high mortality rates were recorded with strain 5310, due to salmonellosis. Thus the presence of strain 5310 in their organs could only be monitored for 10 d (Fig. 3). The increase in counts from 60 min to the value determined 10 d post inoculation was approximately 4 log units. The results obtained with strain 81 were in contrast to those of strain 5310. Although strain 81 was able to reach the spleens and livers of mice inoculated by the intraperitoneal route (Fig. 4), and the counts of it did increase over a period of time, the latter were not of the same magnitude as those of strain 5310. The bacterial count at 60 min post inoculation was approximately log 5 cfu/g in an organ, which is the same as that of strain 5310 at this stage.

TABLE 2 Growth of parent strain 5310 and auxotrophic mutants 102 and 81 in serum

Strains	Saline	% growth increase in	
		*C'-positive serum	C'-inactivated serum
5310	1 604	411	191
102	889	39	4 900
81	1 272	1 750	5 740

* C' = Complement

TABLE 3 LD₅₀-virulence value for mice of *Salmonella* Typhimurium strain 5310 and auxotrophic mutants after intraperitoneal inoculation of mice with 5 log 8 bacteria

Strains	Log ₁₀ of LD ₅₀ -values
5310	4,27
3a	2,22
212	1,74
102	0,59
81	0,91
6	— ^a
17	—

^a Value too low to be determined

TABLE 4 Presence in liver and spleen of parent strain 5310 and mutant strain 81 after oral inoculation of unvaccinated mice

Hours post inoculation	Strains 81		5310	
	Livers	Spleens	Livers	Spleens
1	— ^a	—	+ ^b	+ ^b
3	—	—	+ ^c	+
6	—	—	—	—
24	—	—	+	+ ^b
48	—	—	+	+

^a = *Salmonella* absent

^b = Positive growth after enrichment

^c = *Salmonella* present

TABLE 5 Protection of mice after vaccinations with strain 81 and intraperitoneal challenge by strain 5310 (n = 20)

Mice	% survival after vaccination		
	Intraperitoneal	Subcutaneous	Oral
Vaccinated	88	100	42
Controls	0	5	30

It reached a maximum of log 6 at 3–4 d post inoculation and appeared to remain stable for the following 17–18 d. The mice were then apparently able to eliminate the infection, as isolations of strain 81 could no longer be made from 4 weeks post inoculation.

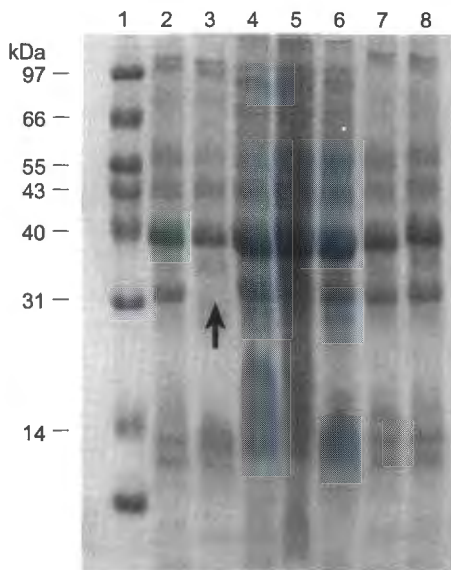


FIG. 2 PAGE of the outer membrane proteins of *Salmonella* Typhimurium strain 5310 and its auxotrophic mutants

Lane 1 = protein molecular mass markers in kDa
 Lane 2 = strain 212
 Lane 3 = strain 81
 Lane 4 = strain 102
 Lane 5 = strain 17
 Lane 6 = strain 6
 Lane 7 = strain 31
 Lane 8 = strain 5310
 Arrow indicates position of protein absent from strain 81

Also, in contrast to the results obtained with strain 5310, no mice died from infection with strain 81 infection although the bacteria were present in their spleens and livers for up to 3 weeks after inoculation.

The mice were well protected against challenge with the virulent parent strain after parenteral vaccination (subcutaneous and intraperitoneal) (Table 5). In the light of the findings that strain 81 was not recovered from the spleens and livers of mice after oral inoculation (Table 4), it was considered that oral vaccination of mice would not be protective and this aspect was not studied further.

Virulence plasmid analysis of the auxotrophic mutants

Plasmid isolation revealed that the mouse virulent parent strain 5310 possessed a high molecular mass plasmid (Fig. 5). It is most probably the same plasmid as the 90 kbp virulence plasmid present in most *Salmonella* Typhimurium strains (Sanderson & Roth 1988). Of the six auxotrophic mutants examined, all except strain 81, contained a similar plasmid. The diffuse lower band visible between 35.9 and 6.9 kbp, (indicated by CHR in Fig. 5) in preparations from all the strains as well as in the marker prepared from *E. coli*, is contaminating chromosomal DNA (Birnboim & Doly 1979; Manning, Baird & Jones 1986; Wray,

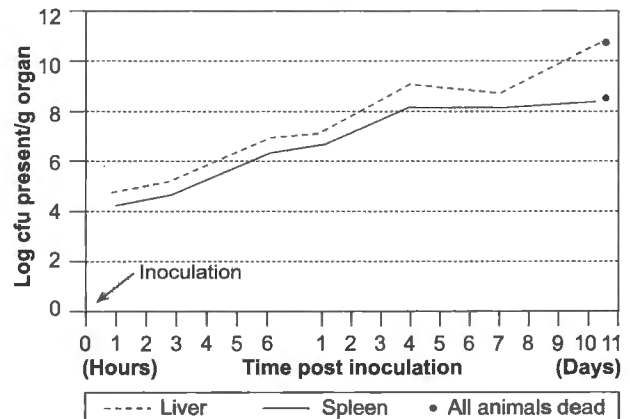


FIG. 3 Counts of strain 5310 in spleens and livers of intraperitoneally inoculated mice

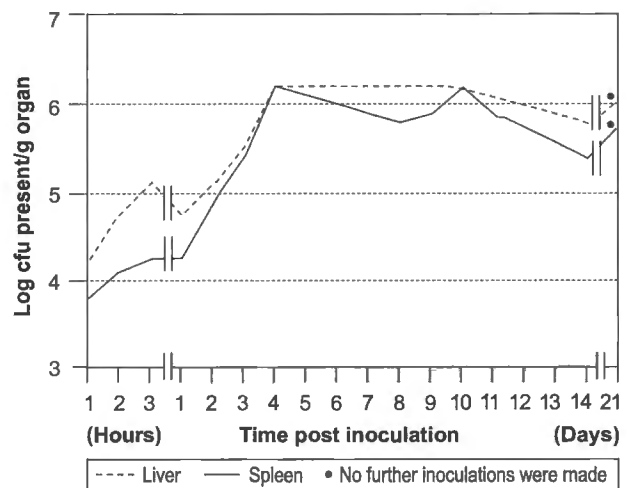


FIG. 4 Counts of strain 81 in spleens and livers of mice inoculated intraperitoneally

McLaren, Parkinson & Beedell 1987; Woodward, McLaren & Wray 1989; Jones & Stanley 1992).

The segment of the 90 kbp plasmid of *Salmonella* Typhimurium to be amplified was determined to be 456 bp. The product of the PCR amplification of strain 5310 was detected on the agarose gel between the 517 bp and 460 bp fragments of the molecular mass markers (Fig. 6). No amplification of the target segment present on the 90 kbp plasmid of the parent strain 5310 occurred when total DNA of the auxotrophic mutant strain 81 was used as target for the PCR. The low molecular mass bands detected below 396 bp were residual primer bands.

DISCUSSION

The high molecular mass plasmid detected in five of the six auxotrophic mutant strains and the prototrophic parent strain 5310, corresponds to the 90 kbp

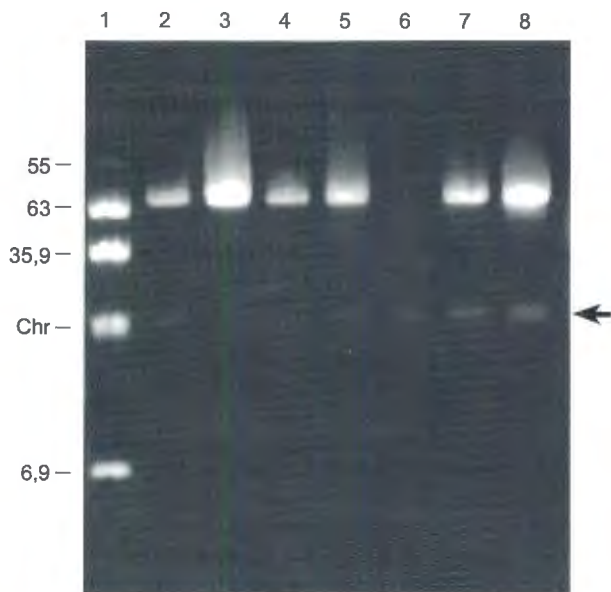


FIG. 5 Presence of high molecular mass plasmids in *Salmonella* Typhimurium strains (indicated by arrow)

MWM = DNA molecular mass markers in kbp
 Lane 1 = parent strain 5310
 Lane 2-7 = its auxotrophic mutant strains
 Lane 2 = strain 3a
 Lane 3 = strain 6
 Lane 4 = strain 17
 Lane 5 = strain 81
 Lane 6 = strain 102
 Lane 7 = strain 212
 CHR = chromosomal DNA

virulence plasmid of *Salmonella* Typhimurium. A segment of the plasmid of strain 5310 was amplified by PCR with a set of primers whose sequences were derived from the published sequence of virulence genes on plasmids of other *Salmonella* Typhimurium strains (Norel *et al.* 1989; Gulig & Chiodo 1990). Further, removal of this plasmid resulted in a strain that was attenuated. The plasmid cured characteristic of strain 81 was confirmed by the sensitive PCR technique, for which total DNA was used as the target in this study. The PCR would have amplified plasmid DNA present in the autonomous state in the cytoplasm, co-integrated in the chromosomal DNA, or present in only a few cells in the culture. The PCR showed that the plasmid loss of strain 81 was complete and that it would be unlikely for this strain to revert spontaneously back to virulence in nature. The attenuated characteristic of strain 81 is thus stable, a requisite for live vaccine strains.

The loss of the virulence plasmid of strain 81 was most probably as a result of the acridine half-mustard treatment used for the generation of auxotrophic mutants and not because of spontaneous loss. The high molecular mass plasmid of *Salmonella* Typhimurium is transferred to descendants in a stable manner, and spontaneous loss is not believed to oc-

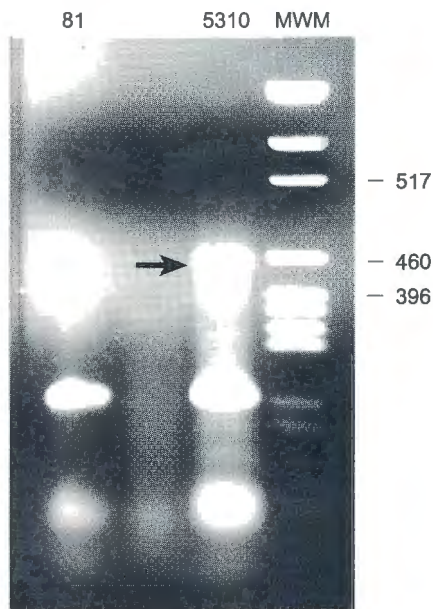


FIG. 6 Determination of the amplification by PCR of a 456 bp segment on the 90 kbp plasmid of *Salmonella* Typhimurium strain 5310 (arrow indicates position of segment). No segment was amplified in the preparation of strain 81
 MWM = molecular weight standards in bp (base pairs)

cur regularly (Spratt, Rowbury & Meynell 1973; Pardon, Popoff, Coynault, Marly & Miras 1986; Sanderson & MacLachlan 1987; Sanderson & Roth 1988; Cerin & Hackett 1989; Tinge & Curtiss 1990). In one study plasmid-free segregants were produced at a rate of less than 10^{-7} per generation. The true plasmid-curing frequency after mutagenesis is unknown, as the absence of the plasmid was determined in only of the 6 auxotrophic mutants, and not in a larger group of cells that survived mutagenesis.

The outer membrane protein which was absent in the preparation of strain 81 had a molecular mass of approximately 30 kDa. The known outer membrane proteins of *Salmonella* Typhimurium which have similar masses are the traT protein, coded for by the virulence plasmid (Rhen & Sukupolvi 1988; Rhen, O'Connor & Sukupolvi 1988; Sukupolvi, Vuorio, Qi, O'Connor & Rhen 1990), and the OmpD and A proteins (Nikaido & Vaara 1987) coded for by the chromosome. However, none of these proteins play a role in virulence. The virulence plasmid also codes for outer membrane proteins that have an apparent role in virulence, but none has a mass similar to that of the protein absent from strain 81 (Rioux, Friedrich & Kadner 1990; Friedrich, Kinsey, Vila & Kadner 1993; Heffernan, Harwood, Fierer & Guiney 1992; Rhen & Sukupolvi 1988; Rhen, O'Connor & Sukupolvi 1988; Sukupolvi *et al.* 1990), Gulig 1990; Sukupolvi, Riihonen, Taira, Saarilahti & Rhen 1992; Hackett, Wyk, Reeves & Mathan 1987). Therefore, the protein which is absent from strain 81 is not believed to be

one of these proteins. It is not known whether curing resulted in the loss of this protein.

The difference in virulence for mice between the parent strain 5310 and the plasmid-cured strain 81, appears to be two-fold:

- The isolation of strain 81 from the livers and spleens of orally dosed mice was impaired when compared to that of strain 5310.
- Strain 81 had a lowered multiplication rate in the livers and spleens of the mice and was not able to kill mice with the doses used.

Other workers have had similar results working with other plasmid-cured strains of *Salmonella* Typhimurium (Jones, Rabert, Svinarich & Whitfield 1982; Hackett, Kotlarski, Mathan, Francki & Rowley 1986; Pardon, Popoff, Coynault, Marly & Miras 1986; Gulig & Curtiss 1987; Rhen & Sukupolvi 1988; Hoertt, Ou, Kopecko, Baron & Warren 1989). It has been determined that the presence of the virulence plasmid of *Salmonella* Typhimurium leads to its increased survival in the tissues of the mice (Gulig & Doyle 1993), which may explain the lower isolation rate of strain 81 from the mice. As strain 81 could not be isolated from the livers and spleens of orally dosed mice, it may be that the ability of this strain to translocate from the intestine to deeper tissues was impaired.

Strain 81 proved effective as a live, attenuated vaccine strain against murine salmonellosis when administered by parenteral routes. The protection conferred on mice after parenteral vaccinations was very high (88–100% survival). The mutative treatment used for the production of the auxotrophic marker on strain 81 did not appear to impair the strain's antigenicity. Plasmid-cured strains are still immunogenic, and the presence of the virulence plasmid of *Salmonella* Typhimurium is not required for effective immunization in the mouse. This strain conformed to all the requirements set for a live vaccine strain for the stimulation of humoral as well as cell mediated immunity: it was smooth, carried an auxotrophic marker, was attenuated and persisted for a period (3 weeks) in lymphoid tissues of mice. In a following paper by the same authors the protective effect of vaccination of calves with this plasmid-cured strain of *Salmonella* Typhimurium, will be discussed.

ACKNOWLEDGEMENTS

The very capable technical assistance of Ms Helena Steyn and Mrs Marion Bezuidenhout is gratefully acknowledged.

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