***Salmonella typhimurium thyA* mutants fail to grow intracellularly *in vitro* and are attenuated in mice**

Menno Kok, Elisabeth Bühlmann and Jean-Claude Pechère

Author for correspondence: Menno Kok. Tel: +41 22 7025652. Fax: +41 22 7025702.
e-mail: menno.kok@medecine.unige.ch

Département de
Génétique et
Microbiologie, Faculté de
Médecine, Université de
Genève, 1 Rue Michel
Servet, 1211 Genève,
Switzerland

***Salmonella typhimurium* ATCC14028 readily multiplies in professional phagocytes *in vitro* and is highly virulent in mice. Mutants lacking thymidylate synthase activity (*thyA*) were isolated and shown to be strictly dependent on thymidine monophosphate precursors in the growth medium. The *thyA* mutants were found to be virtually incapable of intracellular growth and survival *in vitro*, both in macrophage-like cell line P338D₁ and in the human epithelial cell line Hep-2, and their virulence was impaired in BALB/c mice. Intraperitoneal immunization of mice with two doses of live *S. typhimurium thyA* provided protection against a challenge with 10³ times the 50% lethal dose of the virulent parent strain.**

Keywords: *Salmonella*, virulence, thymidylate synthase

INTRODUCTION

Salmonella typhimurium is a facultative intracellular organism. Mice infected orally with a virulent *S. typhimurium* strain may develop murine typhoid following bacterial penetration of the intestinal mucosa. The intestinal lymphoid tissues, the Peyer patches, are preferential sites of bacterial entry (Jones *et al.*, 1995; Clark *et al.*, 1996). Salmonellae probably pass the overlying M-cells by transcytosis, to be liberated at the basolateral pole where the bacteria encounter macrophages, the key players of innate immunity, and dendritic cells, B-cells or T-lymphocytes. Virulent salmonellae evade the innate host defence due to an array of stress-response mechanisms (Miller *et al.*, 1989; Foster & Spector, 1995) and may exploit the natural mobility of lymphoid cells to invade distant sites in the reticuloendothelial system such as the draining lymph nodes, liver and spleen. As a consequence, secondary bacteraemia may develop within a week of primary infection.

The capacity of *S. typhimurium* to survive and grow in the endosomal compartment of macrophages *in vitro* correlates strongly with mouse virulence (Fields *et al.*, 1986; Bäumler *et al.*, 1994; Uchiya *et al.*, 1999), underscoring the important role of these cells in host

defence against bacterial infection. Foreign antigens expressed in attenuated salmonellae may be delivered efficiently to the host immune system. A number of promising attempts have been reported to exploit the lifestyle of this bacterium for vaccination purposes (Coulson *et al.*, 1994; Verma *et al.*, 1995; Hopkins *et al.*, 1996; Nayak *et al.*, 1998). *Salmonella thyA* mutants have also been tested in this context and were found not to be fully attenuated (Smith & Tucker, 1976; Nnalue & Stocker, 1987; Curtiss *et al.*, 1988).

We further examined the behaviour of *S. typhimurium thyA* mutants, which lack the enzyme thymidylate synthase (EC 2.1.1.45) and are incapable of synthesizing DNA in the absence of precursors of thymidine monophosphate (TMP) in the growth medium (Neuhard & Kelln, 1996). We anticipated that these mutants would be incapable of normal DNA synthesis during intracellular growth, due to the absence of TMP and TMP precursors in the endosomal compartment, and that the bacteria would thus suffer 'thymidineless death' (Medoff, 1972; El-Hajj *et al.*, 1992). If this assumption was confirmed, it would suggest that multiplication of the *S. typhimurium thyA* mutants in the mouse would be limited to the extracellular space. In this report we analyse the persistence of thymidine-requiring mutants of the highly mouse-virulent strain *S. typhimurium* ATCC14028 in cultured epithelial and macrophage-like cells and evaluate their virulence in BALB/c mice.

Abbreviations: i.p., intraperitoneal; HBSS, Hanks basic salt solution; TMP, thymidine monophosphate.

METHODS

Strains, plasmids and growth conditions. All *S. typhimurium* strains used in this study were derived from the highly virulent strain ATCC14028. Spontaneous mutants of *S. typhimurium* with an absolute requirement for thymine or TMP were obtained by selection on Luria-Bertani (LB) plates with 500 mg thymidine l⁻¹ and 50 mg trimethoprim l⁻¹ at room temperature. *thyA* mutants were maintained on LB agar plates supplemented with 300 mg thymidine l⁻¹ (LBT). The stability of the mutations was determined by plating dilutions of overnight cultures on LB agar. *S. typhimurium* ATCC14028 *recA* mutants were obtained by P22 transduction, using strain JR501*recA* (K. E. Sanderson, *Salmonella* Genetic Stock Center, University of Calgary, Alberta, Canada), which contains a Tn10 insertion linked to the mutant *recA* gene, as a donor. Plasmid R751::*thyA*, encoding the *Escherichia coli* thymidylate synthase gene has been described before (Kok, 1995); plasmid pILH1, encoding modified listeriolysin of *Listeria monocytogenes* (Gentschev *et al.*, 1995) was a gift from W. Goebel (Würzburg, Germany). *S. typhimurium* was grown in LB broth or on NCE medium (Davies *et al.*, 1980) supplemented with 10 µM each of FeSO₄, MnCl₂, CoCl₂ and CaCl₂, and 1 µM CuCl₂ and ZnCl₂, 300 mg thymidine l⁻¹ (for *thyA* mutants), and glycerol (1%, w/v) or glucose (0.4%) as carbon and energy sources. Trimethoprim was used at 100 mg l⁻¹, gentamicin at 20 or 100 mg l⁻¹ (as indicated) and ampicillin at 80 mg l⁻¹. All chemicals were highest available purity from Fluka or Sigma.

Bacterial growth was followed by measuring the OD₄₅₀ using a 10 mm quartz cuvette in a Uvikon 810 spectrometer (Kontron instruments). The growth rates and generation times of *Salmonella* mutants in thymidine-free media were followed by taking samples every 15 min during the first 90 min following dilution and plotting the optical density versus time. The thymidine requirements of *S. typhimurium* mutants were determined by growing bacteria overnight in complete NCE medium with 0.4% D-glucose and various amounts of thymidine. The final cell density and efficiency of plating were determined for each growth condition. Under non-limiting conditions, wild-type and *thyA* mutants grew to a cell density of approximately 3 × 10⁹ ml⁻¹. The thymidine requirement was defined as the lowest concentration that did not reduce the viable cell count.

Nucleotide sequence determination of the *S. typhimurium thyA* gene. The *S. typhimurium thyA* coding sequence was amplified with 20-mer oligonucleotides complementary to the 5' and 3' ends of the gene (Washington University Genome Sequence Center) in a PCR reaction using the MWG Biotech Primus 96 plus thermocycler. The 30 cycle reaction was carried out with 1 U DyNAzyme (Finnzymes), 0.2 µg genomic DNA, 200 µM each dNTP, 0.5 µM each primer and 5 µl 10-fold concentrated DyNAzyme buffer supplied by the manufacturer, in a 50 µl reaction. The reaction products were analysed on an ABI Prism 377 DNA sequencer.

Growth and maintenance of *S. typhimurium* in cultured cells. The macrophage-like cell line P388D₁ and human epithelial cell line Hep-2 were obtained from the American Type Culture Collection. P388D₁ cultures were maintained on RPMI-1640 supplemented with 10% heat-treated foetal calf serum (FCS; Gibco-BRL), 100 mg streptomycin l⁻¹ and 100 U penicillin ml⁻¹, at 37 °C under 5% carbon dioxide. Hep-2 cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) supplemented with 0.03% L-glutamine, 10% FCS, 100 mg streptomycin l⁻¹ and 100 U penicillin ml⁻¹. *Salmonella* infection experiments were done in 24-well cell

culture dishes (Costar) containing approximately 2 × 10⁵ cells per well. Before infection, the cell cultures were washed with Hanks basic salt solution (HBSS; Gibco-BRL) and supplemented with 1 ml fresh growth medium without antibiotics.

S. typhimurium was grown overnight under aeration at 37 °C in LB, supplemented, if required, with 300 mg thymidine l⁻¹. The culture was diluted to 2 × 10⁷ c.f.u. ml⁻¹ in fresh LB or LBT medium containing 250 mM KCl and 0.5% KNO₃, and incubated at 37 °C without aeration until a cell density of approximately 5 × 10⁸ c.f.u. ml⁻¹ was reached. Bacteria were collected by centrifugation (1500 g, 2 min), and serial dilutions were prepared in the appropriate growth medium (RPMI or DMEM) prewarmed to 37 °C. For infection, 0.1 ml bacterial suspension was added to each well and the plates were briefly centrifuged (3 min at 250 g) to enhance bacterial adsorption to the cell monolayer. After an incubation of 15 min at 37 °C, the cells were washed with 1 × HBSS and supplemented with 1 ml medium with 10% FCS containing 100 mg gentamicin l⁻¹ to eliminate extracellular bacteria. The gentamicin concentration was reduced to 20 mg l⁻¹ 2 h after infection.

Viable intracellular bacteria, expressed as c.f.u., were quantified by lysing the infected cells with 0.05% Triton X-100 in HBSS and plating serial dilutions (in 15 mM MgCl₂) on LBT agar. The total number of cells per well was determined after trypsin treatment using a haemocytometer. The number of infected cells was determined by serial dilution in RPMI or DMEM medium of trypsinized cells, stored on ice for 15 min prior to dilution. The cells were lysed on LBT agar with 0.05 mM EDTA. The number of c.f.u. thus obtained was arbitrarily considered to be equal to the number of infected cells.

Infection of mice. Five-week-old female BALB/c mice (IFFA CREDO) were used for virulence tests. Bacteria were grown to mid-exponential phase (~3 × 10⁸ c.f.u. ml⁻¹) on LB or LBT, collected by centrifugation (1500 g; 3 min at 4 °C) and resuspended in 1 vol. PBS. Appropriate dilutions in PBS were used to verify the viable cell count (c.f.u.). Groups of five mice were injected intraperitoneally (i.p.) with 0.5 ml bacterial suspension and kept under daily surveillance for 3 weeks.

Mice immunized with live or heat-killed (5 min at 95 °C) *S. typhimurium thyA* were challenged (i.p.) 28 or 95 d after the first immunization with 5 × 10³ wild-type *S. typhimurium* ATCC14028 in PBS and their survival was monitored.

RESULTS

Isolation of *S. typhimurium thyA* mutants

Thymidylate synthase is an essential gene in *de novo* synthesis of TMP (Neuhard & Kelln, 1996). Knock-out mutants of *E. coli* and *Salmonella* grow only on media containing TMP, thymidine or thymine, the latter being converted to thymidine by the enzyme deoxyuridine phosphorylase using deoxyribose 1-phosphate as the source of ribose. Selection of spontaneous *thyA* mutants of wild-type strain *S. typhimurium* ATCC14028 yielded 42 mutants resistant to trimethoprim in the presence of excess thymidine. Forty of these were strictly dependent on thymidine (1 mM) in the growth medium, and could not grow on LB medium, nor on LB supplemented with thymine at concentrations below 5 mM. Interestingly, normal colony-forming ability was recovered on 1 mM thymine/LB plates at 20 °C, suggesting that either

uptake of thymine and/or intracellular conversion of thymine to thymidine by the *deoA* product (Neuhard & Kelln, 1996) might be suboptimal at 37 °C, whereas at low growth temperatures thymine can be used as efficiently as thymidine.

The nature of the mutation was verified by genetic complementation with a plasmid-encoded *E. coli* thymidylate synthase gene (Kok, 1995). All 40 mutants were fully complemented for growth on LB and simple mineral media, and were thus denoted *thyA* mutants. Six of the mutants spontaneously reverted to auxotrophy at frequencies between 7×10^{-5} and 2×10^{-7} ; the remaining 34 mutants were used for further experiments.

All mutants displayed an unstable phenotype with respect to their thymidine requirements. Following their isolation on LBT/trimethoprim plates, at least 5 mM thymine or 1 mM thymidine had to be added to the growth media to maintain viability. However, partial suppression of the thymine/thymidine dependence occurred spontaneously during maintenance of the mutants on LBT plates; the thymidine requirement in NCE medium of each of four independent *thyA* mutants going down to approximately 200 µM. We decided to use the partially suppressed mutants, which we will refer to as 'low-thymidine-requiring *thyA* mutants', for further experiments.

Genetic defect in *thyA* mutants

The *thyA* coding sequences of wild-type *S. typhimurium* ATCC14028 and of several high- and low-thymidine-requiring mutants were amplified by PCR for DNA sequencing. The oligonucleotides used for amplification were complementary to the extremities of the *S. typhimurium* TR7095 *thyA* gene, according to the preliminary genome sequence information available from the Washington University of St. Louis Genome Sequence Center (<http://www.genome.wustl.edu/gsc/bacterial/salmonella.shtml>). The sequence of the 792 bp *thyA* gene from the virulent parent strain used in this study was found to be identical to the sequence of strain TR7095 used in the Washington University genome-sequencing project. Two high-thymidine-requiring mutants, *thyA11* and *thyA12*, were found to have single-nucleotide mutations in the coding sequences at codon positions Lys¹⁴⁰ (GAA → AA) and Trp⁸⁰ (TGG → TAG), resulting in premature termination of the ORFs. No additional mutations in the respective coding sequences were detected in the two corresponding low-thymidine-requiring mutants *thyA111* and *thyA121*.

Growth of *S. typhimurium thyA*

When deprived of TMP precursors, *E. coli thyA* mutants undergo 'thymidineless death' (El-Hajj *et al.*, 1992; Medoff, 1972). We evaluated the behaviour of low-thymidine-requiring *S. typhimurium* mutants *thyA111* and *thyA121* when deprived of TMP precursors *in vitro*. First, we established that starved bacteria, washed and resuspended in 15 mM MgSO₄, remained viable (plating

Fig. 1. Survival of *S. typhimurium thyA* on defined media. *S. typhimurium thyA* was grown on LBT at 37 °C to mid-exponential growth phase, concentrated by centrifugation and resuspended in 15 mM MgCl₂. After 1 h starvation at 37 °C, the bacteria were diluted in pre-warmed mineral media with glycerol (white circles) or glucose (black circles) as sole carbon and energy sources. At 15 min intervals, samples were taken to determine the optical density of the cultures, and at regular intervals bacteria were diluted in 15 mM MgCl₂ to determine the efficiency of plating. Arbitrarily, the efficiency of plating after 1 h starvation in MgCl₂ was set to 1.0. The efficiency of plating was plotted against the number of generations as determined from the increase in optical density.

efficiency > 0.9) for at least 24 h. Next, bacteria starved for 1 h in MgSO₄ at 37 °C were diluted in NCE medium containing glucose or glycerol as the carbon source. Bacterial growth resumed virtually without lag after dilution in fresh medium, and for 3–5 h samples were taken to determine the optical density of the culture and the plating efficiency. The correlation between cell growth on thymidine-free medium and viability is shown in Fig. 1. We observed a simple relationship between the mean number of cell divisions (generations) in thymidineless medium and the efficiency of plating. Apparently, cell death increases exponentially with time, after approximately one cell division of thymidineless growth.

Intracellular survival of *S. typhimurium thyA*

We used the macrophage-like cell line P388D₁ to assess the ability of *S. typhimurium thyA* to survive intracellularly. A monolayer of approximately 5×10^5 cells per 2.0 cm² was infected at a multiplicity of infection of 20 bacteria per cell, and the number of surviving intracellular bacteria was followed versus time (Fig. 2). The number of viable bacteria per infected cell was estimated by serial dilution of intact macrophages and of cell lysates, as described in Methods, and corre-

Fig. 2. Survival of *S. typhimurium* ATCC14028 *thyA* in macrophage-like cell line P388D₁. Bacterial survival was determined in macrophage-like cell line P388D₁. Bacteria were put into direct contact with the cells by brief centrifugation of the cell culture plates. Following 15 min incubation at 37 °C, the extracellular bacteria were removed by washing with HBSS and remaining extracellular bacteria were killed by addition of 100 mg gentamicin l⁻¹ to the growth medium. Intracellular survival of *S. typhimurium* ATCC14028 *thyA* was determined by serial dilution of cell lysates. The correlation between time and intracellular survival was established in four independent experiments. The normalized plating efficiency (the colony count after 1 h intracellular growth was set at 1.0 in each of the four experiments) was plotted against time after infection. The mean values are shown; bars indicate standard deviation. The initial infection rate in these experiments was between 1.3 and 2.1 c.f.u. per infected cell.

sponded to 1.6 ± 0.5 bacteria per infected cell, with $18 \pm 4\%$ of the cells infected by at least one bacterium. In contrast to the wild-type parent strain, the low-thymidine-requiring *thyA121* mutant did not grow intracellularly and was virtually eliminated after 24 h (Table 1). Elimination of ATCC14028 *thyA* was found to be virtually independent of the initial infection dose. Only with extremely high bacterial loads, corresponding to more than 10 live bacteria per infected cell, reduced bacterial elimination and extensive death of macrophages were observed after 16–24 h incubation. Apparently, *thyA* mutants are eliminated quickly in a dose-independent manner after invasion of P388D₁ cells. Whether this was mostly due to ‘thymidineless death’ remained to be established, however.

Three approaches were chosen to address this question. First we added 40 mg thymidine ml⁻¹ to the growth medium during infection, to boost the intracellular pool of TMP precursors and stimulate uptake of thymidine by the macrophage in an endocytosis-dependent or -independent manner. Under these conditions, we found a small but significant ($P < 0.05$) increase in bacterial survival after 24 h (Table 1). Addition of thymidine after bacterial invasion did not change the viability of

intracellular bacteria, suggesting that thymidine had to be taken up along with the infecting bacteria. Another plausible way to escape thymidineless death inside the cell would be by passage of bacteria into the cytoplasm. Although *S. typhimurium* does not seem to have the ability to evade the endosomal compartment (Rathman *et al.*, 1996), recombinants equipped with a functional recombinant listeriolysin do (Gentschev *et al.*, 1995). We observed a significant increase ($P < 0.05$) in intracellular survival of *S. typhimurium thyA* equipped with plasmid pILH1, encoding listeriolysin (Table 1). As a third approach to distinguish bactericidal mechanisms employed by the macrophage-like cell line P388D₁ from thymidineless death, we looked at the elimination of *Salmonella* mutants from the human epithelial cell line Hep-2, which does not mount a significant bactericidal activity. We observed rapid clearance of the low-thymidine-requiring mutants *thyA111* and *thyA121* from these cells, whereas all other attenuated *Salmonella* strains tested multiplied readily (Table 1).

Virulence of *thyA* mutants

In view of the very limited life span of low-thymidine-requiring *S. typhimurium thyA* mutants in the endosomal compartments of cultured cells, we anticipated that these mutants would be strongly attenuated in mice. This assumption was tested in BALB/c mice, which were infected by i.p. injection. The low-thymidine-requiring mutants *thyA111* ($LD_{50} = 8 \times 10^4$ c.f.u.) and *thyA121* ($LD_{50} = 1.1 \times 10^5$ c.f.u.) were found to be attenuated, whereas both wild-type *S. typhimurium* ATCC14028 and the *S. typhimurium thyA111* and *thyA121* mutants complemented *in trans* with the *E. coli thyA* gene efficiently infected and killed BALB/c mice at very low doses ($LD_{50} < 10$ c.f.u.). A single immunization with a sublethal dose of the low-thymidine-requiring *thyA121* mutant provided only partial protection against a subsequent challenge, 4 weeks later, with virulent *S. typhimurium* (Table 2). When boosted with a second dose, protection was improved. We could not induce similar protection by i.p. injection of equivalent amounts of heat-killed bacteria, suggesting that live bacteria are required to induce protective immunity.

Recovery of *S. typhimurium* from infected mice

To verify if either the induction of immunity, with moderate doses of low-thymidine-requiring live *S. typhimurium thyA121*, or killing of mice, with high bacterial doses, were due to reversion *in vivo*, we recovered bacteria from the spleens of i.p. infected mice. The five mice injected with 1.8×10^4 c.f.u. were killed 12 d after infection and bacteria were recovered from spleen homogenates. Four out of five mice that had been injected with 1.2×10^6 c.f.u. (high dose) died within 5 d of infection, at which time the spleens were removed to recover bacteria. From the spleens of mice inoculated with the low dose we recovered between 1.1×10^4 and 2.9×10^4 live bacteria, and from each of the spleens of mice inoculated with a lethal dose, more than 10^6 live

Table 1. Intracellular survival of *S. typhimurium*

ND, Not determined.

Strain	Plasmid or growth condition	Growth in Hep-2 cells*	Growth in P388D ₁ cells*
ATCC14028 (parent)	—	31.8 ± 10.6	2.76 ± 0.51
ATCC14028 <i>recA</i>	—	74.1 ± 25.8	1.13 ± 0.47
ATCC14028 <i>thyA</i>	—	0.058 ± 0.027	0.032 ± 0.021
ATCC14028 <i>thyA</i>	pILH1†	ND	0.134 ± 0.057
ATCC14028 <i>thyA</i>	R751::Tn <i>thyA</i> ‡	ND	2.23 ± 0.34
ATCC14028 <i>thyA</i>	40 mg thymidine ml ⁻¹ added during infection	ND	0.148 ± 0.052
ATCC14028 <i>thyA</i>	20 mg thymidine ml ⁻¹ added after infection	ND	0.027 ± 0.017

* Bacterial survival is expressed as viable bacteria (c.f.u.) recovered after 24 h intracellular growth divided by the number of viable bacteria recovered after 3 h intracellular growth. All experiments were performed at least four times; the mean value ± standard deviation is shown.

† Encodes recombinant listeriolysin (Gentschev *et al.*, 1995).

‡ Encodes *E. coli* thymidylate synthase (Kok, 1995).

Table 2. Immunization of BALB/c mice with *S. typhimurium thyA121*

Immunization with strain	Dose (day)	Challenge (day)*	Surviving mice (survival time)†
ATCC14028 <i>thyA</i>	6.5 × 10 ³ (day 1)	None	3/3
ATCC14028 <i>thyA</i>	6.5 × 10 ³ (day 1)	28	1/3 (11 d)
ATCC14028 <i>thyA</i>	6 × 10 ³ (day 1) and 5 × 10 ³ (day 7)	28	3/3
ATCC14028 <i>thyA</i>	7 × 10 ³ (day 1) and 5 × 10 ³ (day 7)	95	3/5 (4 d)
Heat-killed ATCC14028 <i>thyA</i>	6 × 10 ³ (day 1) and 6 × 10 ³ (day 7)	28	0/3 (6 d)
None	—	28	0/3 (4 d)

* Mice were challenged i.p. with 5 × 10³ *S. typhimurium* ATCC14028.

† Mean for the mice that died, in days following challenge.

bacteria were recovered. In neither case could we identify *S. typhimurium* revertants to prototrophy. We also challenged mice with freshly isolated high-thymidine-requiring mutants. However, when we recovered bacteria from the spleens of these animals, we observed both the high- and low-thymidine-requiring phenotypes in proportions that varied considerably from one animal to another.

DISCUSSION

De novo thymidine synthesis in *E. coli* and *Salmonella* depends on the enzyme thymidylate synthase, which converts uridine monophosphate into thymidine monophosphate at the expense of the cofactor tetra-

hydrofolate (Neuhard & Kelln, 1996). Mutants that lack this enzymic activity are strictly dependent on external sources of TMP precursors, such as thymine or thymidine. Depletion of such external sources results in the so-called 'thymidineless death' (El-Hajj *et al.*, 1992; Medoff, 1972). Partial suppression of the ThyA⁻ phenotype occurred in all the *S. typhimurium thyA* mutants we characterized. The spontaneously occurring unidentified extragenic mutation(s) resulted in reduced TMP-precursor requirements. These mutants, denoted 'low-thymidine-requiring', were used for all subsequent experiments. Theoretically, even at concentrations that reduce the efficiency of plating well below 50 % (60 µM), the amount of thymidine in the growth medium constitutes a large excess over the actual TMP require-

ments of the bacterial population. This suggests that even in low-thymidine-requiring mutants, inefficient channelling of thymidine into the TMP pathway paves the way for the erroneous incorporation of UTP into bacterial DNA, which is believed to contribute to bacterial death (Ahmad *et al.*, 1998). The *in vitro* growth experiments clearly show that bacterial death is an inevitable consequence of TMP deprivation. Whereas starved cells retain nearly 100% viability over at least 12 h, cells grown on defined media rapidly lose their colony forming ability.

We have shown that low-thymidine-requiring *S. typhimurium thyA* mutants cannot survive intracellularly. Interestingly, these mutants are readily eliminated from both phagocytic and non-phagocytic cells. This rapid, complete and dose-independent elimination from cultured cells strongly suggests that intracellular bacteria suffer thymidineless death, a direct consequence of growth in an environment that lacks TMP precursors. Moreover, intracellular survival is stimulated under conditions that appear to make at least some thymidine available to the bacterial cells: bacterial endocytosis in the presence of high concentrations of thymidine and evasion of *Salmonella* into the nutrient-rich cytoplasm.

We found, in agreement with previous reports (Smith & Tucker, 1976; Nalae & Stocker, 1987; Curtiss *et al.*, 1988), the impact of the *thyA* mutation on virulence of *Salmonella* in the mouse to be moderate. This apparent discrepancy between the extremely rapid elimination of intracellular bacteria *in vitro* and the moderate attenuation of *Salmonella thyA* mutants *in vivo*, has also been reported by Watson *et al.* (2000) when comparing bacterial survival in alveolar macrophages with virulence in the pig. We hypothesize that extracellular multiplication *in vivo* may be responsible for the observed residual virulence of *S. typhimurium thyA* mutants, with infected organs such as the spleen providing extracellular sources of TMP precursors at concentrations sufficient to sustain bacterial multiplication.

Whereas the *S. typhimurium thyA* mutant provides a useful tool to study the intracellular behaviour of *Salmonella*, its utility in the development of live-vaccination vectors remains to be established. Although low-thymidine-requiring *thyA* mutants offer some protection of mice against a challenge with the virulent parent strain, at least one additional attenuating mutation would have to be introduced into the *Salmonella* genome to render the strain safe for use as a vaccination vector. However, under these conditions the immunogenicity of the *Salmonella* vector may be seriously compromised. An alternative application of *Salmonella thyA* mutants in vaccine development indirectly depends on its virulence effects. Morona *et al.* (1994) have constructed plasmid vectors encoding *E. coli thyA* as a selectable marker for antigen production in a *Salmonella thyA* background, exploiting the selective advantage of thymidine independence *in vivo* to achieve stable plasmid inheritance.

ACKNOWLEDGEMENTS

The authors would like to thank Drs Goebel and Sanderson for supplying bacterial strains. This work was supported by the Swiss Science Foundation (3100-047073) and by the European Union Biotechnology Program (BIO2CT-93049).

REFERENCES

- Ahmad, S. I., Kirk, S. H. & Eisenstark, A. (1998). Thymine metabolism and thymineless death in prokaryotes and eukaryotes. *Annu Rev Microbiol* **52**, 591–625.
- Bäumler, A. J., Kusters, J. G., Stojilkovic, I. & Heffron, F. (1994). *Salmonella typhimurium* loci involved in survival within macrophages. *Infect Immun* **62**, 1623–1630.
- Clark, M. A., Reed, K. A., Lodge, J., Stephen, J., Hirst, B. H. & Jepson, M. A. (1996). Invasion of murine intestinal M cells by *Salmonella typhimurium inv* mutants severely deficient for invasion of cultured cells. *Infect Immun* **64**, 4363–4368.
- Coulson, N. M., Fulop, M. & Titball, R. W. (1994). *Bacillus anthracis* protective antigen, expressed in *Salmonella typhimurium* SL3261, affords protection against anthrax spore challenge. *Vaccine* **12**, 1395–1407.
- Curtiss, R., III, Goldschmidt, R., Kelly, S., Lyons, M., Michalek, S. M., Pastian, R. & Stein, S. (1988). Recombinant avirulent *Salmonella* for oral immunization to induce mucosal immunity to bacterial pathogens. In *Vaccines: New Concepts and Developments*, pp. 261–271. Edited by H. Köhler & P. T. Loverde. London: Longman.
- Davies, R. W., Botstein, D. & Roth, J. R. (1980). *Advanced Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- El-Hajj, H. H., Wang, L. & Weiss, B. (1992). Multiple mutant of *Escherichia coli* synthesizing virtually thymineless DNA during limited growth. *J Bacteriol* **174**, 4450–4456.
- Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. (1986). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* **83**, 5189–5193.
- Foster, J. W. & Spector, M. P. (1995). How *Salmonella* survive against the odds. *Annu Rev Microbiol* **49**, 145–174.
- Gentschev, I., Sokolovic, Z., Mollenkopf, H.-J., Hess, J., Kaufmann, S. H., Kuhn, M., Krohne, G. F. & Goebel, W. (1995). *Salmonella* strain secreting active listeriolysin changes its intracellular localization. *Infect Immun* **63**, 4202–4205.
- Hopkins, S., Kraehenbuhl, J.-P., Schödel, F., Potts, A., Peterson, D., de Grandi, P. & Nardelli-Haefflinger, D. (1996). A recombinant *Salmonella typhimurium* vaccine induces local immunity by four different routes of immunization. *Infect Immun* **63**, 3279–3286.
- Jones, B., Pascopella, L. & Falkow, S. (1995). Entry of microbes into the host: using M cells to break the mucosal barrier. *Curr Opin Immunol* **7**, 474–478.
- Kok, M. (1995). A new horizon for pBR322: *in vivo* insertion of plasmid fragments into wide host range shuttle vectors. *Nucleic Acids Res* **23**, 5085–5086.
- Medoff, G. (1972). Nucleic acid and protein synthesis during thymineless death in lysogenic and nonlysogenic thymine auxotrophs. *J Bacteriol* **109**, 462–464.
- Miller, S. I., Kukal, A. M. & Mekalanos, J. J. (1989). A two component regulatory system (*phoP* and *phoQ*) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci USA* **86**, 5054–5058.
- Morona, R., Morona, J. K., Considine, A., Hackett, J. A., van den

- Bosch, L., Beyer, L. & Attridge, S. R. (1994).** Construction of K88- and K99-expressing clones of *Salmonella typhimurium* G30: immunogenicity following oral administration to pigs. *Vaccine* **12**, 513–517.
- Nayak, A. R., Tinge, S. A., Tart, R. C., McDaniel, L. S., Briles, D. E. & Curtiss, R., III. (1998).** A live recombinant avirulent oral *Salmonella* vaccine expressing pneumococcal surface protein A induces protective responses against *Streptococcus pneumoniae*. *Infect Immun* **66**, 3744–3751.
- Neuhard, J. & Kelln, R. A. (1996).** Biosynthesis and conversions of pyrimidines. In *Escherichia coli and Salmonella*, pp. 580–599. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Nnalue, N. A. & Stocker, B. A. D. (1987).** Tests of the virulence and live-vaccine efficiency of auxotrophic and *galE* derivatives of *Salmonella choleraesuis*. *Infect Immun* **55**, 955–962.
- Rathman, M., Sjaastad, M. D. & Falkow, S. (1996).** Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. *Infect Immun* **64**, 2765–2773.
- Smith, H. W. & Tucker, J. F. (1976).** The virulence of trimethoprim-resistant thymine-requiring strains of *Salmonella*. *J Hyg* **76**, 97–108.
- Uchiya, K.-I., Barbieri, M. A., Funato, K., Shah, A. H., Stahl, P. D. & Groisman, E. A. (1999).** A *Salmonella* virulence protein that inhibits cellular trafficking. *EMBO J* **18**, 3924–3933.
- Verma, K. N., Ziegler, H. K., Wilson, M., Koahn, M., Safley, S., Stocker, B. A. D. & Schoolnik, G. K. (1995).** Delivery of class I and class II MHC-restricted T-cell epitopes of listeriolysin of *Listeria monocytogenes* by attenuated *Salmonella*. *Vaccine* **13**, 142–150.
- Watson, P. R., Paulin, S. M., Jones, P. W. & Wallis, T. S. (2000).** Interaction of *Salmonella* serotypes with porcine macrophages *in vitro* does not correlate with virulence. *Microbiology* **146**, 1639–1649.
-
- Received 9 August 2000; revised 6 November 2000; accepted 22 November 2000.