In vitro* characterization of intra-generic inhibition of growth in *Salmonella typhimurium

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The intra-generic inhibition of bacterial growth observed previously *in vivo* and *in vitro* with strains of *Salmonella*, *Citrobacter* and *E. coli* was studied *in vitro* using *S. typhimurium* strain F98. There was complete inhibition of multiplication of *S. typhimurium* when it was added to stationary-phase broth cultures of different *Salmonella* serotypes, but only partial inhibition when added to broth cultures of *E. coli*. The degree of inhibition between different mutants of F98 was affected by the numbers of bacteria of the inhibiting strain, but this was not the only factor, since exponential-phase bacterial cells were less inhibitory than stationary-phase cells. The inhibitory effect was produced at temperatures between 20 °C and 40 °C. The complete inhibition of growth observed between F98 mutants was abolished by ampicillin, rifampicin and streptomycin, but not by nalidixic acid. Inhibition was also prevented by separating the two cultures by a dialysis membrane. A *TnphoA* insertion mutant of F98 was produced which did not show inhibition *in vitro* but was still inhibitory *in vivo*. It is suggested that this complete inhibition of bacterial multiplication between organisms of the same genus, which is greater than that produced between organisms from different genera, is mediated by a cell surface protein.

Introduction

During a search for strains of non-pathogenic enterobacteria which might inhibit the colonization of the chicken intestine by *Salmonella typhimurium* (Barrow & Tucker, 1986), it was discovered that colonization of the alimentary tract of newly-hatched chickens by one *Salmonella* strain imparted a considerable degree of resistance to colonization by a second strain (Barrow *et al.*, 1987*b*). This phenomenon has recently been explored as a potential method for preventing colonization by food-poisoning *Salmonella* serotypes, by using precolonization with a *Salmonella* mutant avirulent for chickens and man (Berchieri & Barrow, 1990). Similar studies on the use of microbial interference to prevent colonization by pathogenic bacteria have been carried out with a number of different genera including *Escherichia coli* (Duval-Iflah *et al.*, 1983), *Clostridium difficile* (Borriello & Barclay, 1985) and skin staphylococci (Noble & Willie, 1980; Shinefield *et al.*, 1963). It is possible that the mechanisms of inhibition may be different for each type of organism.

The *in vivo* inhibition of colonization occurring between *Salmonella* strains was found to be genus-

specific, not mediated by killed bacteria and not the result of immunity or bacteriophage activity (Barrow *et al.*, 1987*b*). A similar phenomenon was observed *in vitro* in mixed broth cultures. In this case a 24 h broth culture of a *Salmonella* strain completely inhibited the multiplication of a subsequently inoculated *Salmonella* strain, whereas cultures of other genera allowed some multiplication of the second *Salmonella* strain. The lower level of inhibition produced by other genera is likely to be the result of mechanisms such as oxygen depletion, as suggested by Wilson *et al.* (1983). However, the mechanism of complete inhibition of multiplication occurring between *Salmonella* strains in broth is not understood. Its occurrence between isogenic strains precludes bacteriophage and bacteriocin activity. Oxygen or nutrient depletion are not involved and live bacteria are required (Barrow *et al.*, 1987*b*). Using strains of *S. typhimurium*, the present study attempts to shed some light on this mechanism and to relate it, if possible, to the regulation of bacterial multiplication as it is expressed in stationary-phase bacterial cultures.

Methods

Bacterial strains. *Salmonella typhimurium* F98 is a prototrophic, avian, phage type 14 strain which is highly invasive and virulent for

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chickens (Barrow *et al.*, 1987a) and whose faecal excretion characteristics have been well documented (Smith & Tucker, 1975, 1980). Other *Salmonella* strains mentioned in the experimental design were all isolated from poultry or from cases of human food-poisoning. A prototrophic porcine *Escherichia coli* strain, P4, which did not produce complete growth inhibition against *S. typhimurium*, was used as a control organism where necessary.

For the purposes of differentiating one bacterial strain from another in mixed culture experiments, spontaneous chromosomal mutants resistant to either spectinomycin (Spc^r), nalidixic acid (Nal^r), ampicillin (Ap^r), rifampicin (Rif^r), streptomycin (Sm^r) or both nalidixic acid and spectinomycin (Nal^r Spc^r) were isolated as described previously (Smith & Tucker, 1980). With the exception of the rifampicin-resistant mutant, which was of reduced virulence for chickens, these mutants behaved similarly to the antibiotic-sensitive parent strain in both *in vivo* colonization and *in vitro* growth inhibition experiments (P. A. Barrow, unpublished findings). *TnphoA* insertion mutants of F98 Nal^r were produced as described below. Unless otherwise indicated, bacterial strains were maintained on Dorset egg slopes at 4 °C and were cultured in 10 ml nutrient broth (Oxoid CM1) in a shaking water bath (100 strokes min⁻¹) at 37 °C for 24 h. These usually contained between 5×10^8 and 1×10^9 c.f.u. ml⁻¹.

Construction and analysis of *TnphoA* mutants. Plasmid pRT733 (ori R6K, tra⁻, mob⁺, Ap⁺, Km⁺) containing *TnphoA*, a transposon probe for proteins exported to the cell surface (Manoil & Beckwith, 1985), was transferred from *E. coli* SM10 (Simon *et al.*, 1983) into *S. typhimurium* F98 Nal^r by conjugation on a plate surface (Bradley *et al.*, 1980) as described by Finlay *et al.* (1988). Transconjugants were selected on L-agar containing 25 µg sodium nalidixate ml⁻¹, 45 µg kanamycin sulphate ml⁻¹, and 40 µg 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) ml⁻¹. Colonies which were darker blue than the parent strain were checked for ampicillin sensitivity indicating loss of pRT733.

One *TnphoA* mutant of F98 Nal^r was found to be of interest and was examined, together with F98 Nal^r, in greater detail. Its biochemical properties were tested using the API 20E and API CHO kits (API systems, Montalieu Vercieu, France). The mutant was tested for auxotrophy by serial culture on M9 minimal medium (Sambrook *et al.*, 1989). Whole cell protein and outer-membrane protein (OMP) preparations were prepared as described previously (Hassan *et al.*, 1991) and PAGE of these preparations was carried out in a standard way (Sambrook *et al.*, 1989). The plasmid content of the two strains was examined by the method of Kado & Liu (1981).

Southern blot analysis of the mutant and parent were carried out as follows. Bacterial chromosomal DNA was prepared by a modification of the method described by Maskell *et al.* (1988). The pellet from a 100 ml exponential-phase broth culture was resuspended in 2 ml 25% (w/v) sucrose in ice-cold 50 mM-Tris, 1 mM-EDTA, pH 8.0. The cells were lysed slowly for 5 mins with 1 mg lysozyme ml⁻¹ on ice, followed by 500 µg proteinase K ml⁻¹, 0.1 M-EDTA, pH 8.0, and 2% (w/v) *N*-lauroylsarcosine (Sarkosyl), the mixture being left on ice until lysis was complete, followed by overnight incubation at 50 °C. After phenol extraction and ethanol precipitation, the DNA was resuspended in 10 mM-Tris, 1 mM-EDTA, pH 8.0. Restriction analysis, gel electrophoresis, nick translations and Southern blot analysis were carried out as described by Sambrook *et al.* (1989).

Pho A⁺ fusion products of the two strains were sought with non-reducing PAGE, visualizing phosphatase activity with BCIP as described by Finlay *et al.* (1988).

Phage typing was carried out by Dr L. Ward at the Central Public Health Laboratory, Colindale, London, using a standard set of phages, and by the authors using 17 lysogenic phages, obtained from different *S. typhimurium* strains and used at the routine test dilution (Guinée & van Leeuwen, 1978).

Chickens. These were from a specified-pathogen-free Light Sussex flock maintained at this laboratory. Their sex was not determined. Their rearing conditions and diet were as described by Smith & Tucker (1975).

***In vivo* and *in vitro* tests for growth inhibition.** The methods used were those of Barrow & Tucker (1986) and Barrow *et al.* (1987b). A broth culture (0.1 ml) of the strain tested was inoculated orally into the crop of a group of 10 chickens within 24 h of hatching, and before they were given access to food. Twenty-four hours later they were inoculated orally with 10^5 organisms of *S. typhimurium* F98 Nal^r Spc^r in 0.1 ml. Three days later, the chickens were killed and viable counts of the challenge strain were performed on their caecal contents by the method of Miles *et al.* (1938) using brilliant green agar (Oxoid CM 263) containing nalidixic acid (25 µg ml⁻¹) and spectinomycin (30 µg ml⁻¹). *In vitro* inhibition was tested by inoculating a 24 h broth culture of the strain to be tested with the challenge strain (constant initial concentration in the 24 h broth culture of 10^4 c.f.u. ml⁻¹). This was incubated further at 37 °C in a water bath with shaking, and viable counts of the challenge strain were made at intervals on MacConkey agar (Oxoid CM7) containing an antibiotic to which the challenge strain, but not the test strain, was resistant. In the case of *in vitro* inhibition, the inhibitory effect in the challenge strain was expressed as the log₁₀ change in viable count after 24 h incubation. Degrees of inhibition were compared by Fisher's *t* test.

Experimental design

Inhibition of *S. typhimurium* F98 by *Salmonella* strains. Five antibiotic sensitive strains of *S. typhimurium*, one of which was F98, and individual strains of *S. infantis*, *S. heidelberg*, *S. virchow*, *S. hadar* and *E. coli* P4 were tested *in vitro* against *S. typhimurium* F98 Nal^r. To ensure that the Nal^r mutation *per se* did not increase the susceptibility of F98 to the effect produced by other strains, F98 Spc^r was tested for inhibition against F98 Nal^r and F98 Nal^r was tested against F98 Spc^r.

The effect of bacterial numbers and culture age on inhibition. The effect of the number of bacteria in a culture on its inhibitory effect was tested with serial dilutions, made in nutrient broth, of a 24 h broth culture of F98 Spc^r. These were inoculated with 10^4 c.f.u. ml⁻¹ F98 Nal^r and the growth of this latter strain was measured as described above.

The effect of the age of the broth culture of F98 Spc^r was tested by inoculating 10 ml aliquots of nutrient broth with 10^3 c.f.u. F98 Spc^r and culturing these in a shaking water bath for up to 28 d. At intervals, individual cultures were inoculated with 10^4 c.f.u. ml⁻¹ F98 Nal^r, and the growth of this strain was measured as described above.

Since the inhibitory effect on F98 Nal^r in these cultures was affected by bacterial numbers in addition to culture age, a second experiment was performed in which 750 ml prewarmed nutrient broth was inoculated with 10^4 c.f.u. ml⁻¹ F98 Spc^r and incubated in a rotary incubator for 2 h. Preliminary experiments had indicated that the bacteria would be in mid-exponential growth phase at this time. The culture was centrifuged at 10000 *g* for 10 mins and the pellet resuspended in 5 ml nutrient broth to provide a viable count of approximately 10^9 c.f.u. ml⁻¹. This was inoculated with 10^4 c.f.u. ml⁻¹ F98 Nal^r, and the behaviour of the latter strain was tested as described above.

The effect of temperature on inhibition. The effect of temperature was tested using broth cultures of F98 Spc^r which had been incubated at 20 °C, 30 °C, 37 °C or 42 °C in water baths, with shaking, until they had reached the stationary phase (24 h in all cases). These, and sterile nutrient broths, were inoculated with 10^4 c.f.u. ml⁻¹ F98 Nal^r, and the mixed cultures were reincubated at the same temperatures. Growth of F98 Nal^r was monitored in the usual way.

The effect of antibiotics on inhibition. The effect of selected antibiotics on inhibition was tested. A preliminary experiment was carried out to

assess the effects of sodium nalidixate ($25 \mu\text{g ml}^{-1}$), ampicillin ($100 \mu\text{g ml}^{-1}$), streptomycin ($100 \mu\text{g ml}^{-1}$) or rifampicin ($250 \mu\text{g ml}^{-1}$) on the viable numbers of 24 h broth cultures of antibiotic-sensitive F98. Viable counts were made on both MacConkey agar and freshly made MacConkey agar containing the appropriate antibiotic, at intervals following addition of the antibiotics to the cultures. This was done to ensure that multiplication of antibiotic-resistant mutants did not hinder the detection of a reduction in the viable count of the antibiotic-sensitive strain. In a second experiment, 48 h after the addition of the antibiotics to the cultures of F98, 10^4 c.f.u. ml^{-1} of mutants which were either Nal^r , Ap^r , Sm^r or Rif^r , respectively, were added and their subsequent growth was tested, counting the organisms on MacConkey agar containing the appropriate antibiotic.

Separation of cultures by dialysis membrane. The effect on inhibition of separating the cultures by dialysis membrane was tested by inoculating 5 ml sterile nutrient broth with 10^4 c.f.u. ml^{-1} F98 Nal^r and placing this aseptically in dialysis tubing (5.7 mm diameter) which was then incubated in 150 ml 24 h F98 broth culture. The change in viable numbers of the F98 Nal^r culture was assessed over 24 h. Appropriate controls (see Table 3) were included.

Analysis of *TnphoA* mutants of *S. typhimurium* F98 Nal^r . A bank of 200 *TnphoA* mutants of F98 Nal^r which were darker blue than the parent strain was screened for *in vitro* inhibition of F98 Nal^r Spc^r . Cultures which showed reduced inhibition were also tested *in vivo* and examined for other detectable changes in the cell surface.

Results

Inhibition of *S. typhimurium* F98 by *Salmonella* strains

The $\log_{10}$ changes in the viable numbers of *S. typhimurium* F98 Nal^r after incubation for 24 h in a culture of antibiotic-sensitive F98 and in nutrient broth were (mean with standard deviation from nine tests) $+0.11 \pm 0.19$ and 5.20 ± 0.47 , respectively. This difference was statistically significant ($P < 0.001$). F98 Nal^r also produced complete inhibition of F98 Spc^r . The *E. coli* strain produced the degree of partial inhibition described by other authors (Wilson *et al.*, 1983) and attributed to factors such as oxygen depletion ($\log_{10}$ change of $+3.1 \pm 0.88$, results of four tests). This change was significantly different from that found in sterile nutrient broth and from that produced by F98 Spc^r ($P < 0.001$). The changes in the viable numbers of F98 Nal^r produced by incubation with four other *S. typhimurium* strains and individual strains of *S. infantis*, *S. heidelberg*, *S. virchow* and *S. hadar* were (results from three tests) $\log_{10} +0.08 \pm 0.05$, -0.10 ± 0.07 , -0.10 ± 0.09 , -0.13 ± 0.08 , $+0.15 \pm 0.07$, $+0.04 \pm 0.19$, -0.08 ± 0.16 and $+0.03 \pm 0.17$.

The effect of bacterial numbers and culture age on inhibition

The inhibition of growth of F98 Nal^r by diluted cultures of F98 Spc^r is shown in Fig. 1 which represents the mean

Fig. 1. Inhibition of *S. typhimurium* F98 Nal^r by dilutions of 24 h broth cultures of F98 Spc^r .

Fig. 2. Inhibition of *S. typhimurium* F98 Nal^r by broth cultures of F98 Spc^r sampled at various times during the growth cycle. (●) $\log_{10}$ (viable numbers ml^{-1}) of F98 Spc^r at times after inoculation of nutrient broth. (○) Increase after 24 h in $\log_{10}$ (viable numbers ml^{-1}) of F98 Nal^r after inoculation into aliquots of F98 Spc^r .

of results from three experiments. The degree of suppression of growth of F98 Nal^r decreased as the numbers of F98 Spc^r decreased until a concentration of approximately 10^4 c.f.u. of F98 Spc^r ml^{-1} no detectable inhibition occurred.

The $\log_{10}$ increases over 24 h in the numbers of F98 Nal^r after incubation in cultures of F98 Spc^r that were taken at different stages of the growth curve are shown in Fig. 2, along with the viable numbers of F98 Spc^r . There was an inverse correlation between inhibition and the numbers of F98 Spc^r organisms present in the cultures ($n = 27$ from two experiments, $r = -0.961$). Thus inhibition seemed to commence when the viable numbers of F98 Spc^r had reached 10^4 – 10^5 c.f.u. ml^{-1} although it did not reach a maximum until F98 Spc^r was well into the stationary phase; it began to decrease in the phase of decline, from 14 d onwards.

To test whether the reduced inhibition seen during the

exponential phase was due solely to low bacterial numbers, organisms of F98 Spc^r harvested at mid-exponential-phase were centrifuged and resuspended at high density ($\log_{10}$ 8.94 ml⁻¹). The $\log_{10}$ increase in viable numbers over 24 h of F98 Nal^r inoculated into this culture was 1.3 (mean of two tests), indicating that high numbers alone are not sufficient to produce complete inhibition and that cells produced different amounts of the inhibitory principle at different stages of growth. Centrifugation and resuspension in fresh broth had no effect on the inhibitory activity of a stationary phase culture (results not shown).

The effect of temperature on inhibition

The inhibition of F98 Nal^r by cultures of F98 Spc^r was studied at different temperatures, all of which allowed F98 Spc^r to reach stationary phase after 24 h incubation in broth. The $\log_{10}$ changes in the numbers of F98 Nal^r ml⁻¹ after 24 h incubation in mixed culture at 20 °C, 30 °C, 37 °C and 42 °C were $+0.19 \pm 0.06$, 0.10 ± 0.52 , 0.23 ± 0.18 , and 0.20 ± 0.34 , respectively. The $\log_{10}$ increases in F98 Nal^r numbers ml⁻¹ in sterile nutrient broth at these temperatures were 4.53 ± 0.18 , 5.04 ± 0.40 , 4.96 ± 0.45 and 4.80 ± 0.27 at all four temperatures. When the $\log_{10}$ changes in the viable count of the challenge strain after incubation in the F98 Spc^r cultures were compared with those made in nutrient broth, P was <0.01 for all four temperatures.

The effect of antibiotics on inhibition

The minimum inhibitory concentration (MIC) values of ampicillin, streptomycin, nalidixic acid and rifampicin

Table 1. Effect of antibiotics on *S. typhimurium* F98

Antibiotics were added to 24 h broth cultures, which were sampled as indicated, and counted after plating. All determinations were made on MacConkey agar, with (+) or without (-) the same antibiotic used in the broth culture. Results are means of two experiments.

Log ₁₀ (viable numbers ml ⁻¹) <i>S. typhimurium</i> F98 after addition of:								
Time after addition (h)	Nalidixic acid (25 µg ml ⁻¹)		Ampicillin (100 µg ml ⁻¹)		Rifampicin (250 µg ml ⁻¹)		Streptomycin (100 µg ml ⁻¹)	
	+	-	+	-	+	-	+	-
0	<1.0	8.8	<1.0	8.9	<1.0	8.5	<1.0	8.7
2	<1.0	8.8	<1.0	8.3	<1.0	7.9	<1.0	8.6
4	<1.0	8.8	<1.0	7.8	<1.0	9.2	<1.0	8.2
6	<1.0	8.8	<1.0	7.9	1.0	9.2	<1.0	8.2
24	<1.0	8.8	<1.0	5.5	4.6	8.4	<1.0	7.8
48	<1.0	8.9	<1.0	4.3	8.5	7.7	<1.0	7.5

Table 2. Inhibition of growth of antibiotic resistant mutants of *Salmonella typhimurium* F98 by broth cultures of the antibiotic-sensitive parent strain in the presence of antibiotics

Antibiotics were added to 24 h broth cultures of *S. typhimurium* F98, followed 48 h later by 10^4 c.f.u. ml⁻¹ antibiotic-resistant mutants. Results are means from two experiments.

Antibiotic used	Mutant resistant to:	Log ₁₀ change in viable count ml ⁻¹ of F98 mutant over 24 h
Ampicillin (100 µg ml ⁻¹)	Ampicillin	3.8
None	Ampicillin	0.2
Nalidixic acid (25 µg ml ⁻¹)	Nalidixic acid	0.4
None	Nalidixic acid	-0.2
Rifampicin (250 µg ml ⁻¹)	Rifampicin	4.2
None	Rifampicin	0.3
Streptomycin (100 µg ml ⁻¹)	Streptomycin	3.4
None	Streptomycin	0.1

for F98 were 0.49, 7.8, 1.95 and 3.9 µg ml⁻¹ respectively. The MIC values for the F98 strains resistant to these individual antibiotics were 250, 500, 1000 and 250 µg ml⁻¹, respectively. The results of the initial experiment, in which the effect of selected antibiotics on the viable numbers of F98 in 24 h broth cultures was studied are shown in Table 1. Only ampicillin had a marked effect on F98, although this was not apparent for at least 6 h. Streptomycin produced a small reduction in numbers over 48 h and rifampicin and nalidixic acid produced no detectable effect at all. Antibiotic-resistant organisms were only detected following rifampicin treatment. These appeared at 6 h and increased rapidly in numbers up to the end of the experiment.

The effect of the same antibiotics on the inhibition by F98 of appropriate antibiotic-resistant mutants of F98 is shown in Table 2. Inhibition of F98 Nal^r occurred in the presence of nalidixic acid. Complete inhibition of growth of F98 Ap^r, F98 Rif^r and F98 Sm^r was, however, abolished by the presence of ampicillin, rifampicin and streptomycin, respectively. Protein synthesis and an intact envelope structure therefore appear to be essential for complete inhibition of multiplication to occur.

Separation of F98 cultures by dialysis membrane

When small numbers of organisms of the challenge strain F98 Nal^r were separated from an overnight culture of F98 by a dialysis membrane a reduced inhibition of multiplication of F98 Nal^r was obtained, similar to that observed when cells of F98 Nal^r were introduced into a culture of *E. coli* (Table 3). Appropriate controls of single cultures inside or outside the dialysis tubing or of mixed *Salmonella* cultures outside the tubing behaved as expected. Reduced inhibition less than that produced by

Table 3. Effect on growth inhibition of *Salmonella typhimurium* F98 Nal^r of separating the two strains with a dialysis membrane

Strain outside dialysis membrane	Strain inside dialysis membrane	Log ₁₀ increase in viable count of F98 Nal ^r over 24 h
None*	F98 Nal ^r	5.48 ± 0.39 ^a
F98 Nal ^r †	None	5.38 ± 0.27 ^b
F98 sens/F98 Nal ^r ‡	None	0.30 ± 0.17 ^c
F98 sens§	F98 Nal ^r	3.21 ± 0.86 ^d
<i>E. coli</i> P4/F98 Nal ^r ‡	None	3.50 ± 0.65 ^e

* 10⁴ c.f.u. ml⁻¹ F98 Nal^r added aseptically to dialysis tubing placed in 150 ml sterile nutrient broth.

† 10⁴ c.f.u. ml⁻¹ F98 Nal^r added directly to 150 ml sterile nutrient broth.

‡ 10⁴ c.f.u. ml⁻¹ F98 Nal^r added directly to 150 ml 24 h nutrient broth culture of either antibiotic-sensitive parent strain F98 or *Escherichia coli* P4.

§ 10⁴ c.f.u. ml⁻¹ F98 Nal^r added aseptically to dialysis tubing placed in 150 ml 24 h nutrient broth culture of antibiotic-sensitive parent strain F98.

|| Statistical significance of comparisons: *a* vs *d*, *P* < 0.001; *a* vs *c*, *P* = 0.002; *a* vs *e*, *P* < 0.001; *d* vs *e*, *P* = 0.351.

the *E. coli* strain was not expected because other factors such as oxygen depletion (Wilson *et al.*, 1983) would still be active.

Inhibition by and analysis of a *Tnpho A* mutant of F98 Nal^r

Of 200 colonies of F98 Nal^r which were darker blue than the parent strain, only one produced a reduced inhibition of multiplication of F98 Nal^r Spc^r, similar to that produced by *E. coli*. The log₁₀ increases in numbers of F98 Nal^r Spc^r over 24 h in cultures of F98 Nal^r, *E. coli* P4, the *Tnpho A* mutant (designated AB145) and in sterile nutrient broth, were 0.27 ± 0.29, 3.4 ± 0.78, 3.33 ± 1.11 and 4.93 ± 0.12, respectively. The statistical significance (*P* values) in the differences in the changes in viable count of F98 Nal^r Spc^r when incubated with AB145 and when incubated with *E. coli* P4, F98 Nal^r and with nutrient broth, were 0.46, 0.001 and 0.117. The value for the comparison of the change in viable count of F98 Nal^r Spc^r produced by incubation in the culture of *E. coli* P4 and in nutrient broth was *P* = 0.065.

Southern analysis of chromosome DNA from F98 Nal^r and from the mutant AB145, when digested with *EcoRV* and probed with radiolabelled pRT733 (Fig. 3), indicated that the insertion had occurred in a 1.4 kb fragment since *TnphoA* is 7.6 kb in size (Finlay *et al.*, 1988). When examined for plasmid content, both strains possessed a single 85 kbp plasmid. No *phoA* fusion products were detected in the mutant. There was no apparent difference in the protein profiles obtained by

Fig. 3. Autoradiograph of Southern blot from a gel of separated chromosomal fragments from *S. typhimurium* F98 Nal^r and *TnphoA* insertion mutant AB145, digested with *EcoRV* and probed with ³²P-labelled pRT733. Lane 1, F98 Nal^r; lane 2, F98 Nal^r mutant AB145. The positions of molecular mass markers (M) are indicated on the left. The marker is a *HindIII* digest of λ DNA.

PAGE of whole-cell soluble and outer-membrane proteins using a silver stain (results not presented); neither was any difference detected between the two strains in their biochemical activity, pattern of carbohydrate fermentation or sensitivity to a number of bacteriophages. Both strains were prototrophic.

When tested *in vivo* in newly hatched chicks, strain AB145 was as inhibitory as the parent strain. The log₁₀ viable counts of F98 Nal^r Spc^r in the caecal contents of chicks administered F98 Nal^r, AB145 or nothing within 24 h of hatching, followed by F98 Nal^r Spc^r 24 h later were (median with range in parenthesis) <2.0 (<2.0–3.3), <2.0 (<2.0–4.2) and 7.4 (6.2–8.4). Strain AB145, reisolated from chickens at the end of the experiment, was still resistant to kanamycin.

Discussion

As found previously (Barrow *et al.*, 1987), stationary phase cultures of *S. typhimurium* and other *Salmonella* serotypes, but not *E. coli*, inhibited the multiplication of small numbers of *S. typhimurium* organisms when

inoculated into these cultures. The inhibition was not related to the use of *Nal^r* mutants as indicator strains, since *Spc^r* mutants were also inhibited.

Inhibition by cultures in the stationary phase may have been a reflection of both the large numbers of organisms present and characteristics peculiar to this stage of the life cycle. Bacterial numbers, or rather density, were important, since no inhibition was detected when the cell density was less than 10^4 – 10^5 c.f.u. ml⁻¹. Early exponential-phase cultures, when concentrated to the numbers normally present in stationary phase, were not as inhibitory as organisms in stationary phase, suggesting that bacterial cell density was not the only important factor.

The results of previous work (Barrow *et al.*, 1987) suggested that inhibition was not mediated by an extracellular substance secreted into the medium, unless this was very labile, because supernatants obtained by centrifuging broth cultures were not inhibitory. One alternative is that inhibition is mediated through physical contact between individual bacterial cells in the culture. The experiments with antibiotics suggested that the production of the inhibitory principle was not affected by prevention of DNA synthesis with nalidixic acid. However, ampicillin, which prevents cross-link formation in the construction of peptidoglycan by the bacterial cell, and thus affects the integrity of the bacterial envelope, did abolish inhibition. Streptomycin and rifampicin, both affecting protein synthesis, also eliminated inhibition. Rifampicin specifically affects RNA polymerase which also plays an integral role in the initiation of DNA replication (Meyenburg & Hansen, 1989). The hypothesis is thus that intra-generic inhibition of bacterial multiplication is mediated by a bacterial surface protein, and that some form of physical bacterium–bacterium interaction is involved. The latter is also suggested by the lack of inhibition when the two strains were separated by dialysis tubing. Although this does not preclude mediation via a high-molecular-mass molecule released into the broth culture, it seems unlikely that the bacterial cells would synthesise a large, labile molecule continuously and release it into the environment for this purpose.

The *TnphoA* insertion mutation can be used to identify proteins which are transported to the bacterial cell surface (Manoil & Beckwith, 1985) and has been used recently to study surface proteins involved in the invasion of eukaryotic cells by *S. cholerae-suis* (Finlay *et al.*, 1988). In the present study one mutant of *S. typhimurium* F98 *Nal^r* did not produce *in vitro* inhibition of multiplication, suggesting that a surface protein may indeed be involved. However, no differences were detectable between the protein profiles of the parent and the mutant using one-dimensional gels. Neither was

alkaline phosphatase activity detectable in surface preparations made from the mutant. Such mutants were also described by Finlay *et al.* (1988). One explanation for this anomaly is that the insertion of *TnphoA* actually occurred in the signal sequence of the target gene, such that export of alkaline phosphatase to the cell exterior did not occur, although this would not easily explain the initial isolation of mutant AB145 as a colony which was darker in colour than the parent strain.

Although intra-generic inhibition of bacterial multiplication occurs between *Salmonella* strains *in vivo* and *in vitro*, Barrow *et al.* (1987b) did suggest that the mechanisms involved might be different. The fact that AB145 did not produce this inhibition *in vitro* but did so *in vivo* suggests that this might be the case. Additional factors present *in vivo*, such as differences in intestinal colonization ability, may be involved which are not expressed *in vitro* and vice versa.

How signals received at the bacterial surface might affect replication is unclear, but they could be accommodated into either of the two models for replication control, namely the dilution of an inhibitor or the accumulation of an initiator (Meyenburg & Hansen, 1989). These authors suggest that a number of factors interact with the origin of replication, *ori C*, and that this and replication complexes, the replisome, can be isolated in association with bacterial membranes (Jacq *et al.*, 1983; Kusano *et al.*, 1984; Wolf-Watz, 1984), although this may conceivably be an artifact of preparation. The fact that the inhibition of bacterial multiplication is related to replication initiation is supported by the inhibitory effect of rifampicin on both.

Further progress in elucidating the mechanisms of *in vitro* and *in vivo* expression of genus-specific inhibition of bacterial multiplication might be made by the examination of more *TnphoA* mutants. The purification of the inhibitory principle would also assist in this and would allow its assessment *in vitro* and *in vivo*.

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References

- BARROW, P. A. & TUCKER, J. F. (1986). Inhibition of colonization of the chicken alimentary tract with *Salmonella typhimurium* by pre-treatment with strains of *Escherichia coli*. *Journal of Hygiene* **96**, 161–169.
- BARROW, P. A., HUGGINS, M. B., LOVELL, M. A. & SIMPSON, J. M. (1987a). Observations on the pathogenesis of experimental *Salmonella typhimurium* infections in chickens. *Research in Veterinary Science* **42**, 194–199.
- BARROW, P. A., TUCKER, J. F. & SIMPSON, J. M. (1987b). Inhibition of colonization of the chicken alimentary tract with *Salmonella typhimurium* by gram-negative facultatively anaerobic bacteria. *Epidemiology and Infection* **98**, 311–322.

- BERCHIERI, A., JR & BARROW, P. A. (1990). Further studies on the inhibition of colonization of the chicken alimentary tract with *Salmonella typhimurium* by pre-colonization with an avirulent mutant. *Epidemiology and Infection* **104**, 427–441.
- BORRIELLO, S. P. & BARCLAY, F. E. (1985). Protection of hamsters against *Clostridium difficile* ileocaecitis by prior colonization with non-pathogenic strains. *Journal of Medical Microbiology* **19**, 339–350.
- BRADLEY, D. E., TAYLOR, D. E. & COMEN, D. R. (1980). Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K12. *Journal of Bacteriology* **143**, 1466–1470.
- DUVAL-IFLAH, Y., CHAPPUIS, J. P., DUCLUZEAU, R. & RAIBAUD, P. (1983). Intra-specific interactions between *Escherichia coli* in human newborns and in gnotobiotic mice and piglets. *Progress in Food and Nutrition Science* **7**, 107–116.
- FINLAY, B. B., STARNBACH, M. N., FRANCIS, C. L., STOCKER, B. A. D., CHATFIELD, S., DOUGAN, G. & FALKOW, S. (1988). Identification and characterisation of *Tnpho A* mutants of *Salmonella* that are unable to pass through a polarized MDCK epithelial cell monolayer. *Molecular Microbiology* **2**, 757–766.
- GUINÉE, P. A. M. & VAN LEEUWEN, W. J. (1978). Phage typing of *Salmonella*. *Methods in Microbiology* **11**, 157–191.
- HASSAN, J. O., MOCKETT, A. P. A., CATTY, D. & BARROW, P. A. (1991). Infection and reinfection of chickens with *Salmonella typhimurium*; bacteriology and immune responses. *Avian Diseases* (in the Press).
- JACQ, A., KOHIYAMA, M., LOTHER, H. & MESSER, W. (1983). Recognition sites for a membrane-derived DNA binding protein preparation in the *E. coli* replication origin. *Molecular and General Genetics* **191**, 460–465.
- KADO, C. I. & LIU, S. T. (1981). Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology* **145**, 1365–1373.
- KUSANO, T., STEINMETZ, D., HENDRICKSON, W. G., MURCHIE, J., KING, M., BENSON, A. & SCHAECHTER, M. (1984). Direct evidence for specific binding of the replicative origin of the *E. coli* chromosome to the membrane. *Journal of Bacteriology* **158**, 313–316.
- MANOIL, C. & BECKWITH, J. (1985). *TnphoA*: a transposon probe for protein export signals. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 8129–8133.
- MASKELL, D. J., MORRISSEY, P. & DOUGAN, G. (1988). Cloning and nucleotide sequence of the *aro A* gene of *Bordetella pertussis*. *Journal of Bacteriology* **170**, 2467–2471.
- MEYENBURG, K. & HANSEN, F. G. (1989). Regulation of chromosome replication. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, 2nd edn, pp. 1555–1577. Edited by F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter and H. E. Umbarger. Washington DC: American Society for Microbiology.
- MILES, A. A., MISRA, S. S. & IRWIN, J. O. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene* **38**, 732–749.
- NOBLE, W. C. & WILLIE, J. A. (1980). Interactions between antibiotic producing and non-producing staphylococci in skin surface and sub-surface models. *British Journal of Experimental Pathology* **61**, 339–343.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn, vols. 1–3. Cold Spring Harbor NY: Cold Spring Harbor Laboratory.
- SHINEFIELD, M. R., RIBBLE, J. C., BORIS, M. & EICHENWALD, H. F. (1963). Bacterial interference: its effect on nursery acquired infections with *Staphylococcus aureus* I, II, III, IV. *American Journal of Diseases of Childhood* **105**, 646–682.
- SIMON, R., PREIFER, U. & PUHLER, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Bio/Technology* **1**, 784–789.
- SMITH, H. W. & TUCKER, T. F. (1975). The effect of antibiotic therapy on the faecal excretion of *Salmonella typhimurium* by experimentally infected chickens. *Journal of Hygiene* **75**, 275–292.
- SMITH, H. W. & TUCKER, J. F. (1980). The virulence of *Salmonella* strains for chickens; their excretion by infected chickens. *Journal of Hygiene* **84**, 479–488.
- WILSON, G. S., MILES, A. A. & PARKER, M. T. (1983). *Topley and Wilson's Principles of Bacteriology, Virology and Immunity*, 7th edn. London: Edward Arnold.
- WOLF-WATZ, H. W. (1984). Affinity of two different regions of the chromosome to the outer membrane of *E. coli*. *Journal of Bacteriology* **157**, 968–970.