Active but nonculturable cells of *Salmonella enterica* serovar Typhimurium do not infect or colonize mice

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The possibility that nonculturable cells of a normally culturable bacterial pathogen may constitute a source or reservoir for infective disease was investigated. In multiple experiments and with careful attention to the statistical limitations of the assays used, *Salmonella enterica* serovar Typhimurium cells rendered nonculturable by carbon and nitrogen stress in the presence of chloramphenicol were administered orally and intraperitoneally to over 300 female BALB/c mice. Neither infection nor colonization was detected in these studies, even when active but nonculturable (ABNC) cells, as defined by the Kogure cell elongation assay, were present in the inoculum. Doses of ABNC cells exceeding the oral and intraperitoneal LD₅₀ values by 3.5 and 2 orders of magnitude, respectively, were administered. It was concluded that ABNC cells of the salmonella strains used could not be considered potentially infective and that their detection in samples from material being evaluated as a potential source or reservoir of infection by the Kogure test does not specifically represent an infective hazard.

Keywords: viable but nonculturable, viability testing, infectivity

INTRODUCTION

The recognition of sources and reservoirs of bacterial pathogens is a central issue in the control of infectious diseases. Until the 1980s this was achieved by isolation of the pathogen concerned from appropriate sample material. Two developments, the establishment of molecular detection methodologies and the proposal that some bacteria may enter a 'viable but nonculturable' (VBNC) state, require us to re-evaluate this approach. Molecular detection methods can reveal the presence of nucleotide sequences or antigens in diverse sample materials. Their application has revealed a distribution of enteric pathogens that was not detected by culture methods within an epidemiologically defined population (Gaudio *et al.*, 1997). However, without special modifications (e.g. Holmstrom *et al.*, 1999; Sheridan *et al.*, 1998), such methods cannot reveal the

physiological state of the detected bacteria and, as far as we are aware, no studies have defined molecular signals that can indicate their infective potential.

The VBNC hypothesis proposes that some bacteria that are normally readily culturable may enter a state in which they cannot be recovered by standard culture methods and may, nonetheless, retain 'viability' (Barer *et al.*, 1993; Roszak & Colwell, 1987; Xu *et al.*, 1982). This 'viability' has been recognized by various cellular tests of activity and integrity and by a limited number of instances in which a return to culturability has been demonstrated *in vitro* or after animal passage (Cappelier *et al.*, 1999a, b; Kell *et al.*, 1998; Oliver & Bockian, 1995; Bovill & Mackey, 1997; Whitesides & Oliver, 1997; Ravel *et al.*, 1995; Stern *et al.*, 1994; Nilsson *et al.*, 1991; Saha *et al.*, 1991). Many major pathogens, including *Vibrio cholerae* (Colwell, 1996; Xu *et al.*, 1982), *Campylobacter jejuni* (Bovill & Mackey, 1997; Rollins & Colwell, 1986), *Helicobacter pylori* (Gribbon & Barer, 1995; Shahamat *et al.*, 1993) and *Salmonella enterica* (Desmonts *et al.*, 1990; Roszak *et al.*, 1984), are claimed to be able to enter a VBNC state. While the terminology used, the underlying processes involved and

Abbreviations: ABNC, active but nonculturable; CCN, chloramphenicol-treated carbon/nitrogen-stressed; MCCN, minimally stressed CCN; i.p., intraperitoneal(ly); MPN, most probable number; TCC, total cell count; VBNC, viable but nonculturable.

some of the reported data remain controversial (Barer *et al.*, 1998; Bloomfield *et al.*, 1998; Bogosian, 1998; Kell *et al.*, 1998; Oliver, 1999), the possibility that temporarily nonculturable bacteria may be a source or reservoir of infection clearly exists.

Transmission and establishment of a bacterial pathogen from a source to a potential host requires that the pathogen be in an appropriate physiological state, the definition of which remains incomplete. The proposal that some bacteria may enter a physiological VBNC state raises the possibility that pathogens that cannot be detected by conventional culture in potential sources or reservoirs of infection may, nonetheless, be able to initiate infection.

Examples in which culture methods failed to detect the pathogen in likely sources or reservoirs of infection which were positive by molecular biological or immunological methods include cholera (Brayton *et al.*, 1987), campylobacteriosis (Palmer *et al.*, 1983; Pearson *et al.*, 1993) and shigellosis (Gaudio *et al.*, 1997). Although some methods, such as the detection of RNA by RT-PCR (e.g. Holmstrom *et al.*, 1999; Sheridan *et al.*, 1998), provide a step towards defining the relationship between molecular detection signals and infective potential, the application of these approaches has been very limited.

In this study we have extended a well-standardized and quantitative animal infection system (Hormaeche, 1979) to address directly the issue of whether nonculturable cells of culturable bacterial pathogens have infective potential. Our primary concerns have been to prepare cells that were rendered nonculturable by physiologically defined stresses, that the bacterial cellular phenotypes present in animal inocula should be specified in terms of active and inactive components, and that the precision of the infectivity assays should be statistically defined. Colony-forming ability and broth-dilution recovery (most probable number counts) were used to assess culturability and cellular activity was determined by a modified Kogure cell elongation assay (Kogure *et al.*, 1979). The latter demonstrates the capacity of nonculturable cells to elongate in response to low nutrient levels and exposure to a quinolone antimicrobial. We used the same nutrient conditions for the broth recovery and the activity test. Thus, where activity was detected we could be confident that a proportion of the cells inoculated into the broth-dilution test had the capacity for limited growth in the same medium.

Several reports concerning the infectivity of nonculturable bacteria have used animal systems for which there is limited background experience. Therefore we chose *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and the female BALB/c mouse model for infection studies because we wanted to present our data against the existing extensive background of studies that have used this quantitative infectivity assay. Both oral and intraperitoneal administration were used since both have been reported to support resuscitation of nonculturable bacteria (Jones *et al.*, 1991; Oliver & Bockian,

1995). Moreover, the C5 strain of *S. typhimurium* is known to have an intraperitoneal LD₅₀ in the BALB/c system of 1–10 organisms and therefore provides a very sensitive detection system.

The primary aims of this study were: (1) to determine the infective potential of nonculturable cells in the system described and (2) to determine whether active but nonculturable (ABNC) cell counts provide an indication of such retained infectivity. The latter is an important practical issue since the detection of activity remains the principal means by which putative viability and, by implication, infectivity has been determined previously. Our experiments were also designed to determine the value of the Kogure activity assay used here as a means of assessing the hazards posed by detected nonculturable cells. Finally, the use of terms such as viability and death in this field remains controversial (Barer *et al.*, 1998; Bloomfield *et al.*, 1998; Bogosian, 1998; Kell *et al.*, 1998; Oliver, 1999). Where possible we avoid the use of such interpretative terms herein and adhere to a published set of definitions for all potentially ambiguous terms (Barer & Harwood, 1999).

METHODS

Bacterial strains and media. *S. typhimurium* C5 (Hormaeche, 1979) has been extensively investigated in virulence studies (Hormaeche *et al.*, 1995). *S. typhimurium* C5Nx^r, a spontaneous nalidixic-acid-resistant derivative of C5 (Maskell *et al.*, 1987a), was used in the majority of studies. Both strains were cultured in Luria–Bertani broth (LB, 10 g tryptone l⁻¹, 10 g sodium chloride l⁻¹, 5 g yeast extract l⁻¹) and maintained on LB agar. LB broth was diluted 1/10 with double-distilled H₂O to provide 10% (v/v) LB. Sodium mannitol selenite (SMS) broth (4 g sodium biselenite l⁻¹, 19 g mannitol selenite broth base l⁻¹; Oxoid) was used as an enrichment medium for salmonella. For isolation and differentiation of *Salmonella* spp., xylene lysine deoxycholate (XLD) agar (Oxoid) was used. In experiments using *S. typhimurium* C5Nx^r, both SMS broth and XLD agar contained nalidixic acid (50 µg ml⁻¹).

Generation of chloramphenicol, carbon and nitrogen (CCN)-stressed and minimally CCN (MCCN)-stressed populations of *S. typhimurium*. Nonculturability was induced by subjecting the test strains to carbon/nitrogen stress in the presence of chloramphenicol (CCN stress) as described previously (Nwoguh *et al.*, 1995). Briefly, test strains were grown overnight in LB broth at 37 °C on a rotary shaker (200 r.p.m.), diluted into 100 ml LB broth to an OD₆₀₀ of 0.05 and grown to an OD₆₀₀ of 0.2. This dilution process was repeated twice and the final growth period extended to OD₆₀₀ 0.3. These exponential-phase cells were harvested, washed three times with phosphate-buffered saline pH 7.3 (PBS) and resuspended in PBS containing chloramphenicol (100 µg ml⁻¹), to a final density of approximately 10⁸ cells ml⁻¹. The resultant preparations were divided into 50 ml volumes and were maintained statically in 100 ml Duran bottles at 37 °C. The initiation of CCN stress was defined as *T*₀ and subsequent daily intervals as *T*₁, *T*₂, *T*₃, etc. Control populations for determining the effects of CCN stress were made by resuspending exponential-phase cells in chloramphenicol-free PBS.

Minimally stressed CCN (MCCN) cultures were defined as cultures with dissociation between colony-forming and total

cell counts of 5–7 log₁₀ units. This was the minimum level at which inocula could be prepared with confidence that no colony-forming cells were present. The aim was to produce inocula in which some cells that had only just become nonculturable were present.

Colony-forming unit (c.f.u.) counts. Samples were decimally diluted in PBS and 20 µl dropped in six replicates on pre-dried LB agar plates. When counts fell below the detection limit of 50 cells ml⁻¹, 200 µl samples were spread onto five plates to reduce the detection limit to 1 c.f.u. ml⁻¹.

Total cell counts (TCCs). Total cell counts were determined by microscopic enumeration in a Thoma counting chamber (Weber Scientific International). Samples were diluted and fixed with 4 % (v/v) formaldehyde buffered to pH 7.2 with solid K₂HPO₄ (Koch, 1994); samples were diluted further with 0.1 M HCl before filling the counting chamber. Replicate samples were counted using phase-contrast microscopy. All 256 squares of the counting chamber were counted.

Most probable number (MPN) 5-tube method. Samples were decimally diluted down to 10⁻¹⁰ in 1.8 ml volumes of 10 % (v/v) LB broth; five replicates of the dilution series were used. The bijou bottles were held statically at 37 °C and scored for turbidity after 24 h; further changes in turbidity were recorded up to 2 weeks. MPN tables (Department of Health and Social Security, 1969) were then used to derive the MPN of organisms in the original samples.

Kogure elongation test. ABNC cells were identified by a modified Kogure elongation test (Barcina *et al.*, 1995; Kogure *et al.*, 1979). Cells were incubated overnight at 37 °C in 10 % (w/v) LB containing 0.5 µg ofloxacin ml⁻¹. Following incubation, cells were immobilized onto aminopropylsilane-coated coverslips as previously described (Barer, 1991). The resulting monolayer was fixed for 30 s with 4 % (v/v) paraformaldehyde and stained with 0.1 % (w/v) acridine orange. The distribution of cell lengths was determined for treated samples by analysis of digitized phase-contrast images as previously described (Whiteley *et al.*, 1996, 1998) and compared to the mean cell length of the corresponding control population that had not been exposed to ofloxacin. Cells were scored positive for activity if their cell lengths were twice the mean cell length of the control population (Bogosian *et al.*, 1998). In the results reported here this represented 3–4 standard deviations from the control mean; thus less than 0.1 % of the control population would be expected to be scored positive by this criterion. It should be noted that Kogure determinations were not generally done on samples containing culturable cells (e.g. MCCN cells) because elongation was so extensive in some cells that accurate counts could not be achieved and because cell replication took place in control (ofloxacin-free) preparations. Where assays were done on such samples, 10-fold dilutions were performed prior to incubation and counts determined on the first dilution at which filamentous cells did not obscure the result (see Fig. 1).

Animal studies. Inocula for baseline LD₅₀ studies and controls used subsequently to confirm the infective potential of culturable cells were prepared with cells harvested in the exponential phase and maintained in PBS at 37 °C for 18–24 h. In initial studies with the C5 strain we observed that the LD₅₀ values of 24 h stationary-phase cells were not distinguishable from those obtained with the 24 h PBS-maintained cells. The doses were estimated on the basis of TCCs. Inocula of CCN-stressed cells were prepared on the basis of c.f.u. counts relating to samples taken on the previous day. The cells were

Fig. 1. Response to CCN stress of C5 and C5Nx. Δ , C5 TCC; $\blacktriangle$, C5 c.f.u.; $\square$, C5Nx TCC; $\blacksquare$, C5Nx c.f.u. Kogure elongation counts for C5Nx are represented by the vertical bars.

harvested at 15000 g for 10 min at 37 °C, washed with PBS to remove residual traces of chloramphenicol, and resuspended in PBS. TCCs were determined and cell concentrations adjusted to the required dose; further decimal dilutions were performed in PBS. C.f.u. and MPN counts were performed on the inocula.

For experiments determining the infectivity of ABNC, a nonculturable population was required. When CCN cells were nonculturable (<1 c.f.u. ml⁻¹ for 5 days), cells were harvested, washed with PBS and resuspended in 10 % (w/v) LB or PBS. Total cell counts were determined, and the cell concentration adjusted to the required dose. The inoculum was incubated statically at 37 °C for 24 h prior to further decimal dilutions with 10 % (w/v) LB broth or PBS. C.f.u. counts, MPN counts and Kogure elongation test were performed to characterize the inocula.

Female inbred BALB/c mice (Harlan UK), aged 8–10 weeks and with a mean weight of 25 g, were used. Groups of five mice were dosed either orally or intraperitoneally (i.p.) with 10-fold serial dilutions of the test organism. Control mice were inoculated with either PBS or 10 % (w/v) LB depending on the experiment. Mice were housed five per cage, and water and food were provided throughout the duration of the experiment. For oral challenge, mice were lightly anaesthetized with fluorothane and 0.2 ml of inoculum was administered intra-gastrically via a gavage needle. Four days after oral challenge the bowel tissue and cage litter were examined for the test organism by SMS enrichment and direct culture. Bowel tissue was placed in SMS broth and homogenized (Silverson Machines) and cage litter was resuspended at 10 % (w/v) in SMS broth. Broths were incubated statically at 37 °C; samples were removed after 24 and 48 h, plated onto XLD agar plates and incubated at 37 °C for 24 h. Mice dosed i.p. (0.2 ml inoculum) were left for 28 days or until signs of infection developed. Livers and spleens were removed from fatally infected mice and were homogenized separately in 10 ml distilled water in a Colworth stomacher (Hormaeche, 1979). The resultant homogenates were decimally diluted in PBS and the numbers of culturable cells determined by pour plating in LB agar (Maskell *et al.*, 1987b). Putative *Salmonella* colonies were screened on XLD agar plates; subsequent H₂S-reducing colonies were tested for positive agglutination reactions with *Salmonella* agglutinating sera (Murex Diagnostics).

All the animal experiments were performed under licence

from the Home Office (UK). Animals were killed by cervical dislocation.

Mixed-culture experiment. *S. typhimurium* C5 and C5Nx^r were used in this experiment. MCCN C5Nx^r cells were generated as described previously and resuspended in 10% (w/v) LB broth. Inocula were characterized by performing MPN counts and the modified Kogure test. *S. typhimurium* C5 was grown to exponential phase in LB broth. Mice were inoculated orally with 0.2 ml volumes of the mixed culture sample (50:50, C5:C5Nx^r) and mortality was recorded for up to 28 days. Liver and spleen samples were removed from fatally infected mice, processed and examined for the presence of the test organisms by culturing in SMS broth (with or without nalidixic acid at 50 µg ml⁻¹), and plating onto XLD plates (with or without nalidixic acid at 50 µg ml⁻¹). Cage litter from all groups was examined (as described) every 3 days for the presence of the test organisms throughout the experiment. Bowel tissue was removed and examined from all mice that did not die within the 28 day period.

LD₅₀ determination. The LD₅₀ of chloramphenicol-treated and untreated *S. typhimurium* C5Nx^r cells was determined on groups of five female BALB/c mice. Briefly, the test organism was grown to exponential phase, washed and resuspended in PBS (pH 7.2), and chloramphenicol added (100 µg ml⁻¹) as required. Following incubation for 24 h at 37 °C, inocula were prepared by washing and resuspending the cells in PBS. The mice were inoculated orally or i.p. with 0.2 ml volumes of decimal dilutions of cells in PBS. Mortality was recorded up to 28 days, and the LD₅₀ determined by the method of Williams (1986), an approach that enables estimation of confidence intervals by the asymptotic ratio test.

RESULTS

Initial animal experiments were performed with *S. typhimurium* strain C5. In later experiments a nalidixic-acid-resistant derivative of this strain, C5Nx^r, was used to facilitate selective isolation. Infective potential (ability to establish overt infection) was predominantly studied with i.p. inoculations; oral inoculations were generally used to investigate capacity for resuscitation of nonculturable cells.

Responses to chloramphenicol, carbon and nitrogen (CCN) stress

Populations of nonculturable cells were prepared using well-defined protocols that had previously been shown to produce active but nonculturable (ABNC) cells using cellular detection of inducible β-galactosidase activity as a means of determining cellular activity (Nwoguh *et al.*, 1995). The responses of exponential-phase populations of strains C5 and C5Nx^r to CCN stress at 37 °C were monitored by c.f.u. counts and total cell counts (TCCs). Fig. 1 shows a representative pattern of decline in a single microcosm of C5Nx^r and C5 over a period of 14 days. While the c.f.u. counts declined by 8 log₁₀ units over this period, there was no discernible change in TCC. When these strains were subjected to carbon and nitrogen stress in the absence of chloramphenicol, c.f.u. counts declined by 3 log₁₀ units over a period of 35 days, after which no further decrease was observed for up to 6 months (data not shown).

LD₅₀ determinations

The numbers of culturable C5Nx^r cells required to cause 50% mortality (LD₅₀) in BALB/c mice orally and i.p. were determined as 4.92 (±0.75) and 1.14 (±0.26) log₁₀ units, respectively; figures in parentheses denote limits for 95% likelihood based confidence intervals (see Methods). LD₅₀ values for strain C5 fell well within these confidence limits (4.89 and 1.18, respectively). To determine whether chloramphenicol exposure affected virulence, C5Nx^r cells were treated with 100 µg chloramphenicol ml⁻¹ in PBS for 24 h prior to administration. The resultant LD₅₀ values for oral and i.p. inoculations were determined as 4.16 (±1.05) and 1.20 (±0.20) log₁₀ units respectively, values that were effectively indistinguishable from those obtained with untreated cells.

I.p. administration of nonculturable minimally CCN (MCCN)-stressed cells in BALB/c mice

Initial experiments explored the infectivity of MCCN-stressed *S. typhimurium* strain C5. BALB/c mice were challenged i.p. with samples containing approximately 200 stressed cells and for which the probability that a single culturable cell was present was <0.01 (by c.f.u. and MPN). All inoculated mice survived for 28 days and did not develop signs of infection (data not shown). Spleens and livers were removed at 28 days but no culturable *S. typhimurium* cells were detected. In contrast, all mice inoculated with the same number of control, c.f.u.-forming cells that had been held in PBS at 37 °C for 24 h died within 5–10 days; the test organism was subsequently recovered from both their livers and their spleens.

The remaining infectivity experiments were performed using strain C5Nx^r. Over a series of experiments, 75 female BALB/c mice were challenged i.p. with doses containing 10²–10⁴ MCCN stressed cells. The doses and outcome of one experiment are shown in Table 1 (the results of parallel oral inoculations are discussed below). Total cells inoculated exceeded the LD₅₀ by 0.8–3.8 log₁₀ units and the presence of culturable cells was excluded by MPN counts. All mice inoculated with these doses survived for 28 days and did not develop signs of infection.

I.p. administration of ABNC cells does not cause infections

Colwell *et al.* (1996) incubated nonculturable cells of *V. cholerae* in a low-nutrient medium prior to preparing them for oral dosing in human volunteer studies. To assess the effect of comparable preparations in our studies, nonculturable CCN-stressed cells of C5Nx^r were resuspended in chloramphenicol-free PBS or 10% (w/v) LB; the PBS suspensions were administered immediately while the 10% LB suspensions were maintained at 37 °C for 24 h prior to administration and the number of elongation-positive (ABNC) cells determined on the day of inoculation. The infective potential of cell

Table 1. I.p. and oral administration of MCCN-stressed nonculturable *S. typhimurium* C5Nx^r cells to BALB/c mice

Route	Dilution	Doses administered		Outcome	
		TCC	c.f.u.	MPN + ve*	Mice + ve†
I.p.	0.01	50 100	0	1	0
	0.001	5 010	0	0	0
	0.0001	501	0	0	0
	0.00001	50	0	0	0
Oral	1	5 010 000	39	5	0
	0.1	501 000	4	5	0
	0.01	50 100	0	1	0
	0.001	5 010	0	0	0

* Number of tubes positive for growth at this dilution in 5-tube MPN test.

† Number of mice in which infections were detected (i.p. studies only) and number of mice from which culturable *S. typhimurium* C5Nx^r cells were recovered (oral and i.p. studies). Five mice were inoculated at each dilution to make the studies directly comparable with the MPN test.

Table 2. I.p. and oral administration of *S. typhimurium* C5Nx^r populations with defined ABNC cell content

Route	Dilution	Doses administered						Outcomes			
		TCC		ABNC		c.f.u.		MPN + ve*		Mice + ve†	
		PBS	10 % LB	PBS	10 % LB	PBS	10 % LB	PBS	10 % LB	PBS	10 % LB
I.p.‡	NA	NA	NA	NA	NA	3.3	NA	NA	NA	3	NA
I.p.	1	4000	95 500	48§	759§	0	0	0	0	0	0
I.p.	0.1	400	9 550	5	76§	0	0	0	0	0	0
I.p.	0.01	40	955	0	8	0	0	0	0	0	0
I.p.	0.001	4	96	0	0	0	0	0	0	0	0
Oral‡	NA	NA	NA	NA	NA	330 000	NA	NA	NA	5	NA
Oral	1	117 500 000	9 540 000 000	1445 440§	75 857 757§	0	0	0	0	0	0
Oral	0.1	117 500 000	9 540 000 000	144 544§	7 585 776§	0	0	0	0	0	0
Oral	0.01	117 500 000	9 540 000 000	14 454	758 578§	0	0	0	0	0	0
Oral	0.001	117 500	9 540 000	1445	75 858	0	0	0	0	0	0

NA, Not applicable.

* Number of tubes positive for growth at this dilution in 5-tube MPN test.

† Number of mice in which infections were detected (i.p. studies only) and number of mice from which culturable *S. typhimurium* C5Nx^r cells were recovered (i.p. and oral studies). Five mice were inoculated at each dilution to make the studies directly comparable with the MPN test.

‡ Positive control inoculum.

§ Dose above LD₅₀ for c.f.u. (LD₅₀: i.p. 14 c.f.u., oral 83 000 c.f.u.).

populations containing these ABNC cells was investigated and the results are shown in Table 2 (upper half). None of the mice, including those challenged with doses of ABNC cells that exceeded the LD₅₀ by 0.81–2.01 log₁₀ units, developed signs of infection over the 28 day observation period. MPN counts confirmed the absence

of culturable cells in the inocula. In concurrent control studies, five mice were challenged i.p. with a dose estimated at 3.3 c.f.u. of cells maintained in PBS for 24 h. Three of these mice died over the 28 day observation period and culturable C5Nx^r cells were recovered from their livers and spleens.

Table 3. Oral administration of a mixed culture of *S. typhimurium* C5 (culturable) and C5Nx^r (MCCN-stressed) inoculum to BALB/c mice

Inoculum	Doses administered				Outcome*	
	C5 c.f.u.	C5Nx ^r			C5	C5Nx ^r
		TCC	ABNC	c.f.u.		
C5 + C5Nx ^r	10 ⁶	10 ⁷	4.6 × 10 ⁵	4	5	0
	10 ⁵	10 ⁷	4.6 × 10 ⁵	4	5	0
	10 ⁴	10 ⁷	4.6 × 10 ⁵	4	3	0
	10 ³	10 ⁷	4.6 × 10 ⁵	4	1	0
C5 + C5Nx ^r	10 ⁶	10 ⁴	4.6 × 10 ²	0	5	0
	10 ⁵	10 ⁴	4.6 × 10 ²	0	5	0
	10 ⁴	10 ⁴	4.6 × 10 ²	0	5	0
	10 ³	10 ⁴	4.6 × 10 ²	0	2	0
C5	10 ⁶	—	—	—	5	0
	10 ⁵	—	—	—	5	0
	10 ⁴	—	—	—	3	0
	10 ³	—	—	—	2	0

* Score indicates the number of mice from which *S. typhimurium* C5 and/or C5Nx^r cells were recovered. Tissue and/or litter samples were plated (after selenite enrichment with or without nalidixic acid) onto XLD plates with or without nalidixic acid (50 µg ml⁻¹).

I.p. inoculation clearly provides a more sensitive assay for retained infectivity than oral inoculation. Nonetheless, the ability to establish overt infection by oral inoculation of CCN-stressed nonculturable cells was investigated extensively in the initial phase of the study; the maximum dose administered orally was 8.9 log₁₀ units. However, as no such infections were seen over the 28 day observation period and no bacteria were recovered by culture of litter or organs in any of these experiments (data not shown), it was decided to focus oral inoculation studies on the question of whether nonculturable cells could be resuscitated via gut passage.

Attempted resuscitation of nonculturable MCCN-stressed cells via oral inoculations in mice

In multiple experiments, cells were prepared for inoculation into mice when c.f.u. counts of MCCN-stressed populations of strains C5 and C5Nx^r showed dissociations of 5–7 log₁₀ units against the TCC. In initial experiments strain C5 was used as the test organism and, following selenite enrichment of samples, H₂S-reducing colonies resembling *Salmonella* spp. were observed on several occasions. However, the organisms proved difficult to isolate and we were only able to obtain a pure culture that gave the appropriate biochemical and agglutination reactions for *S. typhimurium* on one occasion. Most H₂S-positive colonies isolated from the samples proved to be *Proteus* spp. Subsequently we used the nalidixic-acid-resistant strain, C5Nx^r, which could be selectively isolated from the background flora.

In three experiments done in parallel with those for the infectivity studies described above (Table 1), doses containing 10³–10⁷ MCCN-stressed C5Nx^r cells were administered orally to 75 female BALB/c mice over three experiments. C5Nx^r cells were not recovered from any of the mice at any of the doses even though the highest doses were 1.4–2.4 log₁₀ units above the LD₅₀ and some of the oral doses contained culturable cells (Table 1).

Attempted resuscitation via oral inoculation using nonculturable inocula containing defined numbers of ABNC cells

We next investigated the possibility that ABNC cells in the inoculum might be capable of resuscitation. Inocula were prepared as described above in relation to Table 2. The doses were calculated with respect to cell elongation determinations to provide inocula of ABNC cells that exceeded the oral LD₅₀ determined previously. Four days following oral challenge, the mice were killed, and bowel tissue and cage litter were examined for the presence of the test organism. Groups of five BALB/c mice were challenged orally; doses and outcomes are indicated in Table 2 (lower half). No cell resuscitation/recovery events were observed in any mice and no growth was observed in any of the MPN tubes relating to the inocula used. In a concurrent control experiment in which five mice were challenged orally with 3.3 × 10⁵ c.f.u. of C5Nx^r cells that had been maintained in PBS for 24 h, colonies were recovered from the bowel tissue of all five mice.

Mixed-culture experiment

It has been reported that culturable cells expressing a 'resuscitation factor' may induce a return to culturability in a temporarily nonculturable population (Kaprelyants & Kell, 1994; Votyakova *et al.*, 1994) and an *in vitro* mixed culture test has been developed in an attempt to exploit this phenomenon (Bogosian *et al.*, 1998). The possibility that culturable cells could facilitate resuscitation of nonculturable cells *in vivo* was explored by preparing inocula containing mixtures of easily distinguishable culturable and nonculturable cells that were administered orally to groups of five BALB/c mice. Mixed preparations of culturable, chloramphenicol-treated *S. typhimurium* C5 cells and MCCN-stressed C5Nx^r cells were prepared and the subsequent recovery of the latter nonculturable cells tested by plating on XLD plates containing nalidixic acid. Two dose levels of C5Nx^r (TCC of 10⁷ or 10⁴ cells) were tested and the ABNC content determined. The dose range of C5 cells (10³–10⁶) was prepared such that it straddled the LD₅₀ for the test organism. A control group inoculated with culturable C5 cells only was also included to assess whether addition of nonculturable C5Nx^r cells affected the outcome. Spleen and liver tissue removed from fatally infected mice, and cage litter from all groups, were examined for the presence of 'resuscitated' nonculturable cells of C5Nx^r over 28 days. Doses and outcomes are shown in Table 3. *S. typhimurium* C5Nx^r was not recovered from any sample and the infectivity of C5 was the same with and without the addition of MCCN-stressed C5Nx^r.

DISCUSSION

We have used closely defined conditions and test procedures that are both widely used and well-standardized to study the hypothesis that apparently nonculturable cells of normally culturable bacterial pathogens may have the capacity to resuscitate (i.e. become culturable) or initiate infections *in vivo*. Although this is not a hypothesis that can be falsified in the sense defined by Karl Popper (Popper, 1972), using nonculturable cells prepared under conditions tested here we have found no evidence to support this hypothesis.

Exponential-phase cells of *S. typhimurium* were rendered nonculturable by maintenance in phosphate-buffered, carbon- and nitrogen-free medium (PBS) in the presence of chloramphenicol (CCN stress). This approach yields substantial populations of intact bacterial cells that retain the capacity to respond to a specific external stimulus with *de novo* transcription and translation to form functional polypeptides (Nwogu *et al.*, 1995). The physiological basis for the cell changes observed in the modified Kogure method used here is not defined (Barer *et al.*, 1993; Kogure *et al.*, 1979), although it is likely that transcription, translation and considerable metabolic activity are required (Barer & Harwood, 1999; Kell *et al.*, 1998). In our studies and in other laboratories, it has been recognized that the capacity to respond in the Kogure test is lost relatively early after

cells are rendered nonculturable compared to other indicators of cell activity or integrity (Joux *et al.*, 1997; Lisle *et al.*, 1999). As far as we are aware, the statistical issues relating to scoring cells positive in this assay have not previously been discussed and we have taken particular care to ensure that our ABNC counts could not be attributable to random variation between test and control populations.

It was noted that, in spite of using the same medium for the ABNC and MPN counts, the latter gave results closely corresponding to the c.f.u. counts ($\pm 0.2 \log_{10}$ units where c.f.u. counts were above the limit of detection) in all the assays relating to the experiments reported here (data not shown). The MPN recovery method in unshaken nutrient-poor medium should avoid the problems of metabolic self-destruction that have been suggested as a basis for some nonculturable phenomena (Barer & Harwood, 1999). Apparently cells capable of elongation in the Kogure test medium used here are not necessarily able to replicate sufficiently to produce visible turbidity in the same medium.

In light of these background considerations, we consider that the inocula we used in our animal experiments comprised unambiguously nonculturable cells and that they contained a subpopulation of cells retaining the maximum level of demonstrable activity (minimum degree of stress-associated decline in activity) so far described for ABNC cells. In this way we sought to maximize the opportunities for resuscitation events that might be reflected by recovery of culturable cells from the gut or organs of test animals or by development of clinically apparent infections.

The well-defined *S. typhimurium*–BALB/c mouse model was used to assess the infective capabilities of nonculturable cells. We found no evidence that nonculturable cells (prepared under conditions tested here) were able to initiate infections by the i.p. or oral routes. This effect was not due to chloramphenicol because antibiotic-treated culturable cells (effectively CCN-stressed for 24 h) were shown to have equivalent virulence to untreated cells. Even when doses of ABNC cells rigorously defined using the Kogure test, and exceeding the i.p. LD₅₀ by two orders of magnitude, were administered, no evidence of infection was found. We have attempted to define the statistical limitations of our system and can assert that, at a 95 % confidence level, an inoculum of 95 000 nonculturable cells, estimated to contain a dose of ABNC cells at least 97-fold above the i.p. LD₅₀ for culturable cells, is not infective.

When our initial studies with MCCN cells did not produce any overt infections we concentrated on attempts to detect culturable salmonella cells in the gastrointestinal tract of orally dosed animals and in the livers and spleens of those that received i.p. inocula. Colonization without overt infection has been reported with previous infectivity studies using apparently nonculturable cells of several enteric pathogens and we were concerned not to miss this (Colwell *et al.*, 1996; Jones *et al.*, 1991). One possible recovery event was observed but

we were not able to repeat this subsequently with either test strain. Amongst nonculturable inocula administered to over 300 mice, this was the only possible recovery event. We emphasize that such occasional events are to be expected on statistical grounds when large numbers of experiments are done.

We detected no resuscitation events even when nutritionally amended (10% LB) cell populations containing ABNC cell doses three orders of magnitude above the oral LD₅₀ were administered (Table 2). Finally in the mixed culturable/nonculturable cell inoculation experiments (Table 3), it could be argued that cell populations that had only recently passed from a culturable to a nonculturable state were given the best possible opportunity to resuscitate with the support of antibiotic-sensitive culturable cells. Again no resuscitation events were detected.

Although the initiating stress may be defined, the underlying basis for transition between culturable and nonculturable states is not established in most studies concerned with 'VBNC' cells. In particular, several authors have argued that ABNC cells may represent a long-term survival strategy while others consider such cells to be in a transient or moribund state. Bogosian *et al.* (2000) have recently shown that the temporarily nonculturable cells of *Vibrio vulnificus* studied by Oliver and colleagues (Whitesides & Oliver, 1997) reflect transient peroxide-sensitive populations elicited by temperature downshift. We have been able to detect similar populations (culturable on catalase- or pyruvate-supplemented medium) in the early phase of decline in response to CCN stress but they were always absent by the times at which inocula for animal experiments were prepared (R. J. Smith & M. R. Barer, unpublished observations). It seems plausible that peroxide-sensitive cells might be recovered by passage through the predominantly anaerobic large intestine. In two recent studies, cells of *S. typhimurium* made nonculturable by UV stress (which also produces oxidative stress) showed no retained infectivity when administered intraperitoneally (Caro *et al.*, 1999; Smith *et al.*, 2000). More recently, Kolling & Matthews (2001) orally challenged mice with starvation-induced or chlorine-induced nonculturable *Escherichia coli* O157:H7 cells. No cells were recovered following passage through the gut.

We recognize that alternative stresses provoking nonculturability might give different outcomes. Similar considerations apply to alternative strains of pathogen and host, and to the choice of recovery media and regimens. While noting that these issues cannot be addressed 'by any finite series of experiments' (Bogosian *et al.*, 1998) we conclude that: (1) we have established a rigorous approach to the recognition of *in vivo* resuscitation events involving defined populations of nonculturable bacterial pathogens; (2) ABNC cells meeting the criteria that others have used to recognize 'viable but nonculturable' bacteria are not infective in our system; (3) the presence of ABNC cells detected by the Kogure method cannot be considered predictive for the infective hazard

posed by the material sampled; and (4) at present, there are no validated means other than culture by which molecular signals obtained from clinically relevant samples can be shown to indicate the presence of infective bacteria. We urge others concerned with the issues addressed here to consider adopting comparable approaches to assessing the infectivity of nonculturable bacteria and hope that this will lead to a more precise view of whether and to what extent temporarily nonculturable bacteria (Barer & Harwood, 1999; Ekweozor *et al.*, 1998) constitute a source and a reservoir for infective diseases.

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