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Bioluminescence as a Reporter of Intracellular Survival of *Bordetella bronchiseptica* in Murine Phagocytes

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The uptake and persistence of *Bordetella bronchiseptica* was characterized in murine phagocytes by using a novel bioluminescence-based reporter system. A mini-Tn*5* promoter probe carrying the intact *lux* operon from the terrestrial bacterium *Photorhabdus luminescens* which allowed measurement of light output without the addition of exogenous substrate was constructed. It was used to create a pool of bioluminescent fusion strains of *B. bronchiseptica*. The internalization and persistence in murine macrophages of a constitutive bioluminescent strain of *B. bronchiseptica* was monitored by luminometry and by fluorescence and electron microscopy. The number of bacteria internalized, in a microfilament-dependent process, by a mouse macrophage-like cell line after 2 h was approximately 1% of the inoculum for several different multiplicities of infection (MOI). At an MOI of <500:1 (bacteria to macrophages), viable numbers of intracellular bacteria declined over a 4-day period. However, at an MOI of ≥500:1, long-term survival was enhanced, with viable bacteria recovered up to 4 days postinfection with little decline in numbers, indicating that a critical population size may have been essential for intracellular persistence. No evidence of macrophage killing by intracellular bacteria was detected over the 4-day period. Intracellular bioluminescent *B. bronchiseptica* organisms in mouse peritoneal cells were detected at 24 and 48 h after intraperitoneal injection of mice. Bioluminescence is shown to act as a convenient real-time technique for monitoring of intracellular survival of *B. bronchiseptica* in vitro and may provide a suitable means for examining the role of long-term intracellular survival of the bacterium in the host.

The bordetellae were, until recently, generally considered to be extracellular pathogens associated predominantly with the respiratory tract of humans, animals, and birds. However, a number of studies have shown that *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* may be found intracellularly within a variety of eukaryotic cells. *B. pertussis* has been reported to invade or be internalized by HeLa (15, 31), CHO (36), and Hep-2 cells (40), human and rabbit cultured primary macrophages (18, 32, 42), and human polymorphonuclear leukocytes (PMN) (12, 28, 45). Invasion of HeLa cells and human respiratory epithelial tissue culture cells by *B. parapertussis* has also been reported (14). The invasive properties of *B. bronchiseptica* have been examined in mouse dendritic cell lines (6, 20, 21), in epithelial cells (44), and in HeLa cells (33, 43, 49), and uptake into murine macrophages has also been reported (1).

Bordetella species synthesize a number of dedicated virulence factors whose expression is regulated by the *bvgA-bvgS* operon (8), and the role of this locus in directing internalization and intracellular survival has been studied. Uptake of *B. pertussis* appears to be a *bvg*-dependent process, as *bvg* mutants have been found to be significantly less invasive in a number of cultured cells (15, 18, 31, 40), but the bacterial factors essential for this process have not been fully characterized. The adenylate cyclase toxin (ACT) of *B. pertussis* has been described as protecting the bacteria against killing by macrophages (32), but others have reported that it induces apoptosis in macrophages (29, 30), although not in PMN (28). In a more recent study, Banemann and Gross (1) found no evidence for induction of apoptosis of macrophages by intracellular *B. pertussis* or *B. bronchiseptica.*

In contrast to *B. pertussis*, uptake of *B. bronchiseptica* has been shown to be *bvg* independent in all cell types examined (1, 20, 44). In studies in which the behaviors of *B. pertussis* and *B. bronchiseptica* have been compared directly, both the wild type and *bvg* mutants of *B. bronchiseptica* were internalized and persisted for 3 days or more in dendritic, epithelial, and macrophage cells, but numbers of intracellular *B. pertussis* organisms decreased gradually over the assay period until none could be recovered (1, 20, 43, 44). Two studies demonstrated that *bvg* mutants of *B. bronchiseptica* had a survival advantage compared to wild-type strains in murine macrophages (1, 44). *B. pertussis* and *B. bronchiseptica* are thus markedly different, having *bvg*-dependent and *bvg*-independent invasion capacities, respectively, and the former appears unable to persist once internalized whereas the latter survives for a period of days. The factors essential for uptake and survival of *B. bronchiseptica* have not been identified, although a recent study showed that *B. bronchiseptica* mutants lacking an acid phosphatase activity, not found in other *Bordetella* species, displayed a significant reduction in levels of intracellular survival after 24 h in a dendritic cell line (6). Urease-negative and nonmotile mutants of *B. bronchiseptica* have also been found to have a reduced capacity to survive intracellularly in HeLa cells (33, 49). The relevance of these in vitro observations to natural infection remains to be confirmed, but intracellular persistence may represent a chronic or quiescent stage in vivo. *B. pertussis* has, in fact, been reported within mouse and rabbit pulmonary macrophages after experimental infection (4, 42) and in pulmonary alveolar macrophages from children with human immunodeficiency virus infection (2).

B. bronchiseptica is among a growing number of pathogenic bacteria, including *Helicobacter* (25) and *Campylobacter* (50) species, which were considered to be exclusively extracellular

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Bordetellae		
B. bronchiseptica 5376	Wild type	37
B. bronchiseptica CBF1	Spontaneous Rif^r mutant of 5376	This work
B. bronchiseptica CBF2	As CBF1 but *xxx*::Tn*5 lux*	This work
B. bronchiseptica CBF3	As CBF1 but *xxx*::Tn*5 lux*	This work
B. avium 4148	Wild type	39
B. avium CAF1	Spontaneous Rif^r mutant of 4148	This work
B. parapertussis 10520	Wild type	NCTC[a]
B. parapertussis CPF1	Spontaneous Rif^r mutant of 10520	This work
E. coli strains		
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lac*ZX74 *galE galK phoA20 thi-1 rpsE rpoB argE*(*Am*) *recA1* (λ*pir*)	23
SM10λ*pir*	Kan^r; *thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (λ*pir*)	23
DH5α	*supE44* Δ*lacU* (ϕ80*lacZ* M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	22
Plasmids		
pT7-3	Amp^r; contains T7 promoter, ϕ10, and the *luxCDABE* genes from *P. luminescens*	46
pUTmini-Tn*5*km	Amp^r; Tn*5*-based delivery plasmid with Kan^r	10
pUTmini-Tn*5*km2	Amp^r; Tn*5*-based delivery plasmid with Kan^r	10
pUTmini-Tn*5*km*lux*	As pUTmini-Tn*5*km but carries the *luxCDABE* genes from *P. luminescens*	This work
pUTmini-Tn*5*km2*lux*	As pUTmini-Tn*5*km2 but carries the *luxCDABE* genes from *P. luminescens*	G. Stewart, University of Nottingham, Nottingham, United Kingdom

[a] NCTC, National Collection of Type Cultures (Central Public Health Laboratory, London, England).

but for which there now exists some evidence of an intracellular phase. Pathogens such as these can be considered weakly invasive, in terms of the numbers of bacteria internalized, compared to facultative intracellular bacteria such as *Salmonella*, *Shigella*, and *Yersinia* species, whose intracellular lifestyles have been relatively well characterized. Investigations of low-level internalization and survival would be enhanced by the development of real-time methods for monitoring the activities of intracellular bacteria within eukaryotic cells, which would also be helpful in isolating noninvasive mutants. For this reason, the internalization and persistence of *B. bronchiseptica* was examined in a murine macrophage-like cell line and in murine peritoneal phagocytes by using bioluminescence as a reporter of bacterial viability. A mini-Tn*5*-based suicide vector which carries the *lux* genes from the terrestrial bacterium *Photorhabdus* (*Xenorhabdus*) *luminescens* (46) was constructed. The *lux* transposon acts as a promoter probe in *B. bronchiseptica* and allowed the generation of a random pool of stable bioluminescent fusion strains. In addition to its potential for study of the regulation of gene expression, this system offers a number of other advantages for monitoring intracellular survival. The intact *lux* operon is carried within the minitransposon, and there is no requirement for the addition of exogenous substrates, which are often toxic to cell lines, to render the bacteria bioluminescent. The *P. luminescens* luciferase is stable at temperatures up to 42°C, in contrast to the low-temperature optima for eukaryotic and other prokaryotic luciferases (17), which is an important consideration when examining host-microbe interactions involving animal pathogens. As bioluminescence is directly related to cellular viability (34), real-time monitoring of light output from internalized bacteria allows semiquantitative estimation of both the numbers of intracellular bacteria and the length of time that they are able to persist.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bordetella* species (Table 1) were grown on Bordet-Gengou (BG) agar (Difco) containing defibrinated horse blood (20% [vol/vol]). Liquid cultures were grown in CL medium (26). *Escherichia coli* strains (Table 1) were grown on Luria-Bertani (LB) agar or in LB broth. Antibiotics were used at the following concentrations: rifampin (Rif), 150 μg/ml; kanamycin (Kan), 50 μg/ml; ampicillin (Amp), 100 μg/ml; polymyxin B sulfate, 50 or 10 μg/ml.

Minitransposon construction. Restriction endonuclease digestion, agarose gel electrophoresis, and isolation of DNA from agarose gels were done by standard methods (41). A 7-kb *Eco*RI fragment bearing the intact *lux* operon (*luxC*, *luxD*, *luxA*, *luxB*, and *luxE*) from *P. luminescens* Hb ATCC 29999 was isolated from plasmid pT7-3 (46) and blunt-ended with the Klenow fragment of DNA polymerase at 25°C for 15 min in the presence of 4 μM deoxynucleoside triphosphates. Plasmid pUTmini-Tn*5*km (10) was linearized with *Not*I and blunt-ended in the same way. Ligation mixes of *Not*I-linearized, blunt-ended pUTmini-Tn*5*km and the 7-kb *Eco*RI *lux* fragment were used to transform the *Escherichia coli* host strain CC118λ*pir* by electroporation (13). Transformants (Kan^r Amp^r) were examined for bioluminescence with a charge-coupled device (CCD) luminescence imager (Photonic Science). Recombinant plasmid pUTmini-Tn*5*km*lux* (Fig. 1) was extracted from host cells with Qiagen Mini-Prep columns, in accordance with the manufacturer's instructions, and used to transform *E. coli* donor

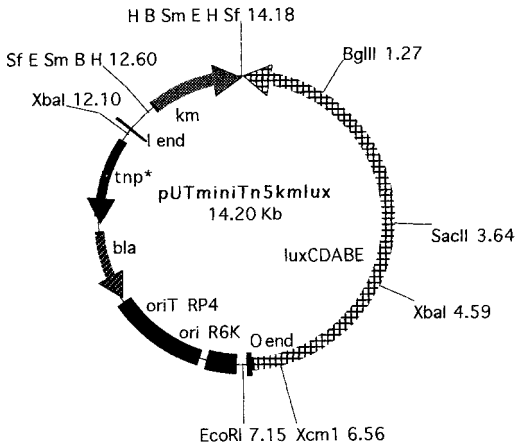

FIG. 1. Construction of the suicide vector pUTmini-Tn*5*km*lux*. A 7-kb *Eco*RI fragment carrying the *lux* operon from *P. luminescens* was excised from pT7-3 and blunt-ended with Klenow fragment. The delivery vector pUTmini-Tn*5*km was digested at its unique *Not*I site and also blunt-ended. Both DNAs were ligated to give the vector pUTmini-Tn*5*km*lux*. Recombinant plasmids in which the *lux* genes were oriented divergently with regard to the kanamycin resistance marker, such that the *lux* genes were transcribed from native promoters upon transfer of the transposon to the host chromosome, were identified. Restriction sites shown: H, *Hin*dIII; B, *Bam*HI; Sm, *Sma*I; Sf, *Sfi*I; E, *Eco*RI.

strain SM10λ*pir* by electroporation. Again Lux$^+$ transformants (Kan^r Amp^r) were isolated by CCD imaging.

Minitransposon mutagenesis. Mutagenesis experiments were adapted from a previously described procedure (23). Exponential cultures of a spontaneous Rif^r mutant of *B. bronchiseptica* 5376 and *E. coli* donor strain SM10λ*pir* (pUTmini-Tn*5*km*lux*) were resuspended in 10 mM $MgSO_4$. Plate matings were performed at 37°C on BG plates for 16 h at a recipient-to-donor ratio of 3:1. Growth was resuspended in 1 ml of 10 mM $MgSO_4$, and 100-μl aliquots were spread on BG selective plates (with rifampin and kanamycin) and incubated overnight at 37°C. Exconjugants (Kan^r Rif^r Amp^s) were picked randomly to gridded BG plates and examined by CCD imaging.

Internalization assay with cultured macrophages. The murine macrophage-like cell line P388D1 was maintained in RPMI 1640 medium (containing fetal calf serum [10%, vol/vol] and 10 mM glutamine) at 37°C in the absence of antibiotics. Approximately 10^5 CFU/ml were seeded in tissue culture plates or flasks and incubated for 48 to 72 h until cell numbers had increased to around 5×10^5 cells/ml. Cell numbers and viability were confirmed by trypan blue exclusion counts. Infections were carried out with suspensions of *B. bronchiseptica* at ca. 5×10^9 CFU/ml which were prepared in RPMI from 16-h cultures on BG plates. After incubation for 2 h at 37°C, cells were washed twice with phosphate-buffered saline (PBS), and extracellular bacteria were killed by resuspension in RPMI supplemented with polymyxin B sulfate (Sigma) to a final concentration of 50 μg/ml and incubation for 90 min. This concentration of polymyxin killed >99.99% of 5×10^9 *B. bronchiseptica* CBF2 organisms over the 90-min period, as established by automated luminometry (see below) and confirmed by plate counts. Cells were washed three times with PBS, the monolayer was disrupted mechanically with a cell scraper, and cells were resuspended in RPMI. For study of intracellular survival times >24 h, macrophages were maintained in RPMI supplemented with polymyxin to a final concentration of 10 μg/ml to ensure that any residual extracellular bacteria were not allowed to grow, and this medium was replaced at 48-h intervals. Where appropriate, macrophages were pretreated before infection for 72 or 24 h with recombinant gamma interferon (IFN-γ) (Promega) at a concentration of 100 U/ml or for 1 h with cytochalasin D (CD) (Sigma) or monodansylcadaverine (MDC) (Sigma) at 0.5 μg/ml and 50 μM, respectively. Viability of infected macrophages was assayed with the CellTiter 96 nonradioactive cell viability assay (Promega) in accordance with the manufacturer's instructions. This assay measures cell viability by assessing the ability of mitochondria in living cells to reduce a tetrazolium dye to formazan. Assays were performed in 96-well trays, and the absorbance at 620 nm, which is directly proportional to the number of living cells, was measured in an enzyme-linked immunosorbent assay plate reader (Anthos Labtech 2001).

Automated luminometry of bacterial survival in cultured macrophages. A Wallac LKB 1251 luminometer running on Multi-Use software (Bio-Orbit Oy, Turku, Finland) was used to measure the light output at 37°C from infected macrophages beginning at 4 h postinfection. This was the time required for internalization of bacteria by macrophages, washing to remove the majority of extracellular bacteria, and killing of residual extracellular bacteria by polymyxin. Duplicate 1-ml suspensions of infected macrophages in RPMI were removed and placed in the luminometer, and light output was monitored, usually for ~3 h. Polymyxin was added to 50 μg/ml, as recording of light emission commenced (t = 0), to ensure that the light emitted was from intracellular bacteria. After 30 min, digitonin, at 100 μg/ml, was added to one of the duplicate samples to lyse the macrophages and expose intracellular bacteria to the polymyxin in the suspending medium. Bacterial killing was indicated by a decrease in light output. Serial dilutions of digitonin-treated and untreated suspensions were plated to LB agar for bacterial counts, and the Lux$^+$ phenotype of recovered bacteria was confirmed by CCD imaging. Killing of inocula by polymyxin at each multiplicity of infection (MOI) was demonstrated with luminometry and confirmed by plate counts.

Automated luminometry of bacterial survival in murine peritoneal exudate cells. Murine peritoneal exudate cells were harvested from normal female BALB/c mice. Duplicate 1-ml samples containing 5×10^5 peritoneal exudate cells in RPMI, or P388D1 cells as controls, were placed in the luminometer. A suspension of *B. bronchiseptica* CBF2, prepared as described above, was added at an MOI of 500:1. After 2 h, to allow for bacterial internalization, polymyxin was added to 50 μg/ml to kill extracellular bacteria. After a further 90 min, digitonin was added to 100 μg/ml to one of the duplicate samples to lyse the eukaryotic cells. Light emission was recorded continuously for ~8 h.

Mouse infection studies. Adult female BALB/c mice were infected intraperitoneally with *B. bronchiseptica* CBF2 at ~10^9 and 10^8 CFU/mouse. Peritoneal exudate cells were recovered at 24 and 48 h postinfection, and two 1-ml samples were placed in the luminometer. Polymyxin was added immediately (t = 0), to 50 μg/ml, to kill extracellular bacteria, and digitonin, to 100 μg/ml, was added to one of the duplicate samples after 90 min. Light emission was recorded continuously for ~8 h.

Transmission electron and fluorescence microscopy. Cultured macrophages and peritoneal exudate cells were harvested by centrifugation at 400 × *g* for 15 min, washed in PBS, and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 6 h. Samples were then rinsed in 0.1 M phosphate buffer (three changes at 10-min intervals) and postfixed in 0.1% osmium tetroxide for 1 h. After two rinses in distilled water for 10 min each, samples were dehydrated in an ethanol series and embedded in Spurr resin. Sections were cut on an LKBIII Microtome, stained in uranyl acetate and lead citrate, and examined with a Zeiss 902 electron microscope.

For fluorescence microscopy of infected cultured macrophages, washed cells were stained with a mixture of green fluorescent nucleic acid stain (SYTO 9) and red fluorescent nucleic acid stain (propidium iodide), supplied in the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes), in accordance with the manufacturer's instructions. Bacteria with intact cell membranes stain fluorescent green, whereas cells with damaged membranes stain fluorescent red. It was also found that macrophages could be differentially stained with LIVE/DEAD *Bac*Light stain; its usefulness as a marker of macrophage viability was confirmed by direct comparison with trypan blue counts (data not shown). Counts of live (green) and dead (red) bacteria or macrophages were carried out with a Vickers M20 Photoplan microscope fitted with a standard fluorescein longpass filter set.

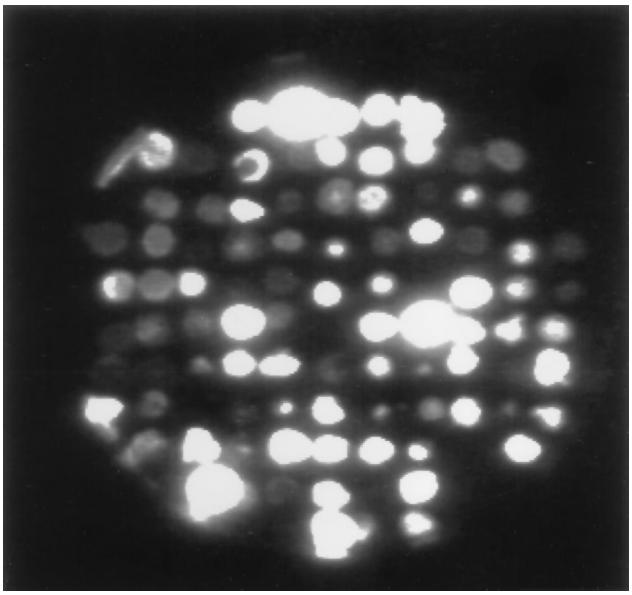

FIG. 2. A representative grey scale CCD image of random *B. bronchiseptica* exconjugants. Twenty colonies arising from each of five independent conjugations were picked randomly to BG agar and incubated for 24 h at 37°C. Photon emission was imaged in a 5-s integration period with a Photonic Science luminescence imager running on Improvision Openlab software.

RESULTS

Isolation of a constitutive bioluminescent strain of *B. bronchiseptica*. The transposition efficiencies in *B. bronchiseptica* CBF1 of the mini-Tn*5* transposons pUTmini-Tn*5*km, pUTmini-Tn*5*km*lux*, and pUTmini-Tn*5*km2*lux* ranged from 4.5×10^{-5} to 5×10^{-5}. The presence of accessory DNA in the transposon, in the form of the *lux* operon, had no effect on the transposition efficiency. Similar frequencies of transposition also occurred in *B. parapertussis* CPF1 and *B. avium* CAF1, at 5×10^{-5} and 5×10^{-6} respectively, although no transconjugants were obtained with *B. pertussis* 18-323. The minitransposon pUTmini-Tn*5*km*lux* was used to generate a pool of 2,000 mutants of *B. bronchiseptica* CBF1 via 10 independent conjugations. As expected with a construct which acts as a promoter probe, levels of expression of bioluminescence varied between mutants, as viewed by CCD imaging (Fig. 2). Expression of bioluminescence in three very bright fusion strains was examined on BG agar and BG agar supplemented with $MgSO_4$ or nicotinic acid, as these stimuli are known to affect *bvg*-dependent regulation of gene expression in vitro in *Bordetella* species. The level of light output was found to be unaffected by the growth medium. Expression of luminescence was also unaffected at low pH (pH 5.5) and in the presence of the superoxide generator methyl viologen. Such signals have been shown

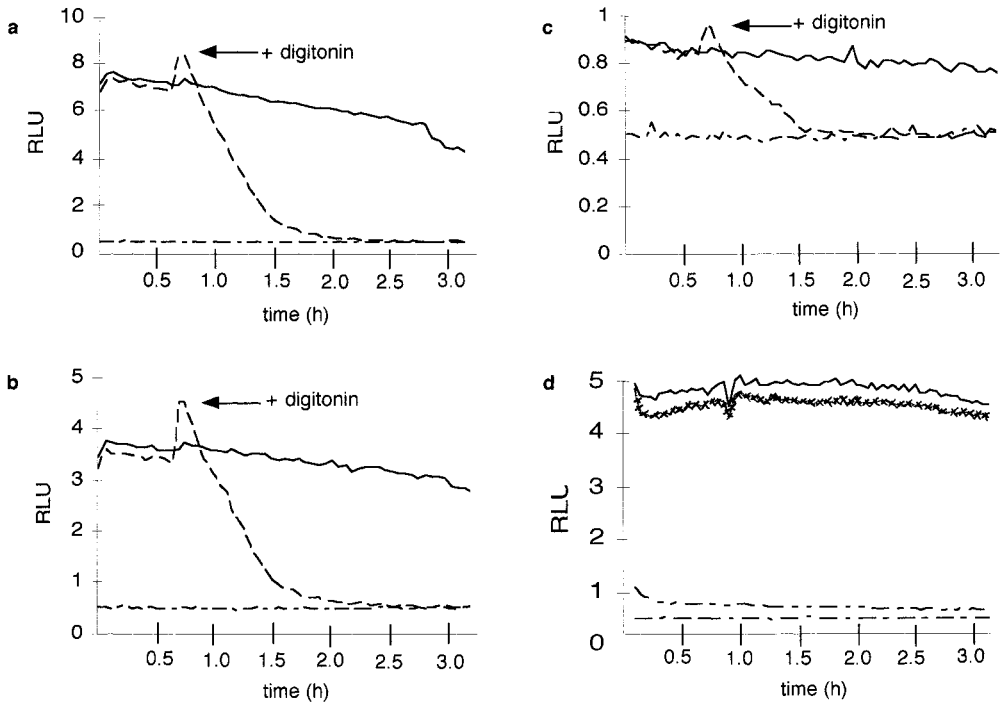

FIG. 3. Real-time analysis of light emission by intracellular *B. bronchiseptica* CBF2 in the murine macrophage cell-line P388D1. Invasion assays were carried out at MOI of 1,000:1 (a), 500:1 (b), and 100:1 (c). In a separate experiment, P388D1 cells were pretreated for 1 h with CD or MDC before infection at an MOI of 500:1 (d). At 4 h postinfection, the infected monolayers were disrupted, cells were resuspended in RPMI supplemented with polymyxin, and light output was monitored continuously for ~3 h. Assays were carried out twice in duplicate for each MOI. Representative luminometer traces are shown. ———, P388D1 cells plus CBF2 plus polymyxin; – – – –, P388D1 cells plus CBF2 plus polymyxin plus digitonin; — — — — —, P388D1 cells only; — – — – — –, P388D1 cells plus CBF2 plus polymyxin plus CD; ********, P388D1 cells plus CBF2 plus polymyxin plus MDC. Bioluminescence is expressed as RLU.

to be important factors in regulating gene expression in other intracellular pathogens. When levels of bioluminescence were measured by automated luminometry, the brightest strain, CBF2, showed light output greater than that found in the *P. luminescens* parent or in *E. coli* DH5α carrying the multicopy plasmid pT7-3. The growth rate of the Lux^+ derivative in CL medium was unaffected compared with the wild-type parent (data not shown). Consequently, the fusion strain CBF2 was chosen to act as a constitutive reporter strain for intracellular survival.

Detection of intracellular bioluminescent *B. bronchiseptica* in cultured macrophages. Measurement of the light output, in relative light units (RLU), from 5-ml suspensions of P388D1 cells which had been infected with *B. bronchiseptica* CBF2 at MOI of 1,000:1 (5×10^8 CFU/ml), 500:1 (2.5×10^8 CFU/ml), and 100:1 (5×10^7 CFU/ml) was monitored after internalization of bacteria by macrophages, washing to remove the majority of extracellular bacteria, and killing of residual extracellular bacteria by polymyxin from 4 h postinfection for a further period of 3 h (Fig. 3a to c). The light outputs of bacterial inocula at 5×10^8, 2.5×10^8, and 5×10^7 CFU/ml were measured as 467, 241, and 55 RLU, respectively, in the absence of macrophages (data not shown). It can be seen from Fig. 3a to c that the final RLU recorded from polymyxin-treated P388D1 cells represented ~1 to 2% of the total RLU recorded from the bacterial inoculum alone. Plate counts confirmed the usefulness of measurement of light output as a semiquantitative estimate of numbers of bacteria internalized, as they were also found to represent ~1 to 2% of the total inoculum (data not shown). As <0.01% of *B. bronchiseptica* CBF2 at $\sim 5 \times 10^9$ CFU/ml survive treatment with polymyxin for 90 min, this level of light output was unlikely to have been due to viable extracellular bacteria. However, it is possible the presence of eukaryotic cells might have interfered with killing of the bacteria by the antibiotic, and so the intracellular location of luminescent bacteria was confirmed in two ways.

First, the mammalian cells were lysed by the addition of digitonin to one of the duplicate samples at 30 min. This led to exposure of intracellular bacteria to the polymyxin in the suspending medium, and bacterial killing was shown by a rapid decrease in light output (Fig. 3a to c). Digitonin alone had no effect on the viability or light output of *B. bronchiseptica* CBF2, as demonstrated by luminometry and confirmed by plate counts (data not shown). In the absence of digitonin, internalized bacteria continued to emit light throughout the assay, demonstrating that they retained viability and that polymyxin did not accumulate within the macrophages.

Second, macrophages were pretreated, before infection with *B. bronchiseptica* CBF2, with CD or MDC to inhibit microfilament-dependent phagocytosis and receptor-mediated endocytosis, respectively. Neither CD nor MDC at the concentrations used had any effect on the viability or light output of *B. bronchiseptica*, as demonstrated by luminometry and confirmed by plate counts (data not shown). Figure 3d shows that when the macrophages were pretreated with MDC, light output and, hence, internalization of the bacteria were largely unaffected. With CD pretreatment, however, light output was marginally above the basal level recorded from uninfected control macrophages. Thus, internalization of *B. bronchiseptica* by macrophages appeared to have been due to microfilament-dependent phagocytosis and was inhibited to >95% in the presence of CD. The small amount of light emitted from CD-treated macrophages originated from internalized bacteria, as addition

TABLE 2. Relationship between MOI, internalization, and persistence of *B. bronchiseptica* CBF2 in P388D1 cells[a]

MOI	Mean (SD) CFU/ml at h postinfection indicated				
	4	24	48	72	96
1:1	4,000 (600)	450 (200)	190 (470)	21 (11)	<10
10:1	5.1×10^4 (900)	3,900 (3300)	460 (210)	9 (10)	<10
100:1	2.2×10^5 (2.1×10^4)	7,600 (2000)	860 (130)	16 (9)	<10
500:1	2.1×10^6 (2.0×10^5)	1.6×10^6 (4.9×10^5)	2.0×10^6 (2.8×10^5)	2.3×10^6 (2.0×10^5)	1.9×10^6 (2.0×10^5)
1,000:1	5.4×10^6 (4.7×10^5)	5.9×10^6 (4.0×10^6)	5.5×10^6 (1.8×10^6)	4.8×10^6 (3.4×10^5)	4.9×10^6 (4.4×10^5)

[a] Macrophages at 5×10^5/ml in 96-well trays were infected at MOI (bacteria to macrophages) of 1:1, 10:1, 100:1, 500:1, and 1,000:1, which represent 5×10^5, 5×10^6, 5×10^7, 2.5×10^8, and 5×10^8 CFU of bacteria/ml, respectively. Assays were done in triplicate.

of digitonin resulted in a rapid decline to baseline levels (data not shown).

A slow decline in light output was evident throughout these luminometry assays, and this was most marked with the highest inoculum. However, when assays were carried out over longer periods (8 h), steady-state levels of light output were reached (data not shown). As seen in Fig. 3a to c, a burst in light output was evident at the point of digitonin addition, possibly as a result of greater oxygen availability to the bacteria released into the suspending medium compared to those in the intracellular environment. Although the intracellular compartment of the macrophage is regarded as an oxygen-limited environment, expression of bioluminescence, an oxygen-dependent reaction, by the intracellular bacteria did not appear to be prevented. However, it is possible that some quenching of light output occurs as a consequence of internalization of bacteria, due in part to oxygen availability, and the numbers of internalized bacteria estimated by light output should be confirmed by plate counts when exact numbers are required.

Effect of MOI on internalization and persistence. In subsequent experiments, the effect of MOI on internalization and survival of *B. bronchiseptica* was examined because the data in Fig. 3a to c had indicated a relationship between inoculum size and numbers of bacteria internalized. P388D1 cells in 96-well trays were infected with *B. bronchiseptica* CBF2 at MOI of 1,000:1, 500:1, 100:1, 10:1, and 1:1, and numbers of intracellular bacteria were determined by plate counts. The numbers of viable bacteria recovered at 4 h postinfection for each MOI represented around 1% of the initial inoculum in each case (Table 2). On continued incubation of the infected, polymyxin-treated monolayers, numbers of intracellular bacteria decreased steadily on a daily basis by around 1 log unit, when macrophages were infected at MOI of less than 500:1, until day 4, when no bacteria were recovered (Table 2). However, in those monolayers infected at MOI of ≥500:1, viable bacteria were still recovered at day 4. In a parallel experiment, light output was detected at 48 h or more postinfection only from suspensions of macrophages infected at MOI of ≥500:1 (Table 3). Light output declined slightly with time over the 96-h period, but there was no significant reduction in bacterial numbers, as determined by simultaneous plate counts. As the macrophages appeared to remain viable throughout the assay period, this may have reflected a reduction in bacterial metabolic activity. Plate counts showed that *B. bronchiseptica* CBF1, a wild-type strain, and CBF3, a strain carrying a Tn*5 lux* insertion at a different site from CBF2, were internalized and persisted in the same manner as did CBF2 (data not shown).

Intracellular survival of *B. bronchiseptica* in activated macrophages. The cell line P388D1 was incubated with the cytokine IFN-γ at 72 or 24 h preinfection, and light output from internalized *B. bronchiseptica* CBF2 was monitored in comparison to that from infected nonactivated macrophages. Activation of the cell line resulted in a decrease in light output from infected macrophages, with the least light emitted from those macrophages incubated with IFN-γ at 72 h preinfection (Fig. 4a). This suggested that activation of the cell line increased the antibacterial ability of the macrophages, but direct quantitation of bacterial survival by plate counts is required to confirm the effect. After an obvious decline during the first 30 min of the assay, light output appeared to stabilize, indicating that *B. bronchiseptica* CBF2 was still able to survive intracellularly in the IFN-γ-treated macrophages, although at a reduced level. Digitonin was added to one of the duplicate samples of treated and untreated macrophages at 30 min, and light output rapidly declined to the basal levels observed in uninfected macrophages (data not shown). A higher light output was initially recorded from IFN-γ-activated macrophages than from untreated macrophages, suggesting either that higher numbers of surviving extracellular bacteria were associated with the cell surface of IFN-γ-treated macrophages or that higher numbers of intracellular bacteria were present at time zero.

Survival and detection of intracellular *B. bronchiseptica* in peritoneal exudate cells. To confirm that the murine macrophage-like cell line P388D1 acts as a representative model for intracellular survival of *B. bronchiseptica* in phagocytes, survival was examined in both P388D1 cells and murine peritoneal exudate cells harvested from uninfected mice. Suspensions of the two cell types at $\sim 5 \times 10^5$ cells/ml were infected with *B. bronchiseptica* CBF2 at an MOI of 500:1, and light output was then monitored continuously for 8 h. After a 2-h period to allow for internalization, polymyxin was added (Fig. 4b) and killing of extracellular bacteria and survival of intracellular bacteria were assayed. As before, digitonin was added to one of the duplicate samples, and light output rapidly declined to the basal levels observed in uninfected macrophages (data not shown). No significant differences were observed in the light outputs from both cell types after polymyxin killing of extra-

TABLE 3. Relationship between MOI, persistence, and light output of *B. bronchiseptica* CBF2 in P388D1 cells[a]

MOI	Mean RLU at h postinfection indicated			
	4	24	48	96
10:1	0.68	0.55	<0.5	<0.5
100:1	1.6	0.9	0.55	<0.5
500:1	8.0	3.6	4.2	2.5
1,000:1	18.0	9.1	5.0	4.2

[a] Ten-milliliter suspensions of macrophages at 5×10^5/ml were infected at MOI (bacteria to macrophages) of 10:1, 100:1, 500:1, and 1,000:1. The RLU of the inocula were 11, 95, 350, and 780, respectively. Suspensions of 1-ml samples or, at an MOI of 10:1, 5-ml samples concentrated in 1 ml of RPMI were assayed by luminometry at the times shown. Individual flasks were used for each MOI and for each time point. Assays were done in duplicate.

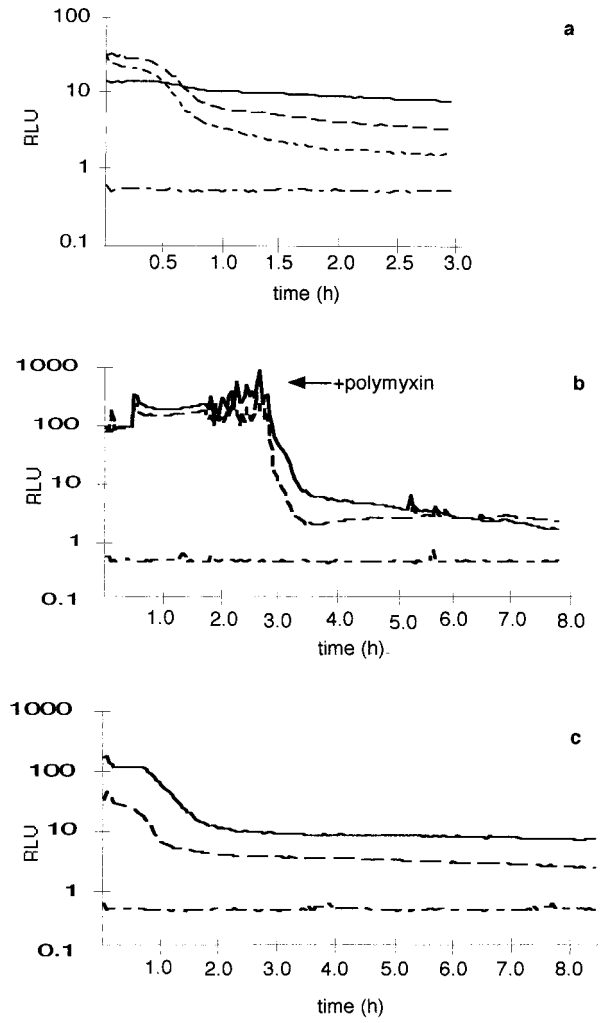

FIG. 4. Light output as a monitor of survival of *B. bronchiseptica* CBF2 in activated P388D1 cells and mouse peritoneal exudate cells. (a) P388D1 cells were incubated with INF-γ at 72 h (– – – – –) and at 24 h (— — —) preinfection or in the absence of IFN-γ (———) and infected at an MOI of 500:1. At 4 h postinfection, light output from disrupted monolayers was monitored continuously for ~3 h. (b) Freshly harvested uninfected mouse peritoneal exudate cells (———) and P388D1 cells (— — —) were placed in the luminometer and infected at an MOI of 500:1. Monitoring of light output commenced immediately and was recorded continuously for ~8 h. After a period of 2 h to allow for bacterial invasion, polymyxin was added to kill extracellular bacteria. (c) Peritoneal exudate cells from mice infected intraperitoneally with ~10^9 CFU were harvested at 24 h (———) and at 48 h (— — —) postinfection and placed in the luminometer. Polymyxin was added immediately to kill extracellular bacteria, and light output was monitored for ~8 h. Invasion assays were carried out twice in duplicate. – — – —, uninfected cells only. Representative luminometer traces are shown. Bioluminescence is expressed as RLU.

cellular bacteria and continuous monitoring over a period of 5 h; it appeared, therefore, that survival rates of intracellular bacteria were similar in the two cell types.

Mice injected intraperitoneally with ~10^9 or 10^8 CFU of *B. bronchiseptica* CBF2 were sacrificed at 24 and 48 h postinfection, and peritoneal fluid was collected in sterile PBS. Light output was monitored in peritoneal fluid samples for a period of 8 h after the immediate addition of polymyxin to the samples to kill extracellular bacteria. Bioluminescence was recorded from peritoneal cells recovered from those mice infected with a dose of 10^9 CFU of bacteria at both 24 and 48 h postinfection (Fig. 4c). Addition of digitonin to one of the duplicate samples resulted in a rapid decline in RLU to the basal levels observed in uninfected macrophages, indicating that light output after polymyxin treatment was from intracellular bacteria (data not shown). Light output was not recorded from peritoneal exudate cells recovered from mice infected at the lower bacterial inoculum at either 24 or 48 h postinfection, suggesting again that a critical population of bacteria may be necessary to establish intracellular persistence under these conditions.

Microscopic analysis of cells infected with *B. bronchiseptica*. The distribution of *B. bronchiseptica* CBF2 associated with P388D1 cells infected at MOI of 500:1 and 100:1 was examined by fluorescence microscopy with LIVE/DEAD *Bac*Light stain. After killing of extracellular bacteria with polymyxin, 48% of macrophages infected at an MOI of 100:1 were found to have live bacteria associated with them, while at an MOI of 500:1, 97% of macrophages had live bacteria associated with them. In general, ≤5 live bacteria were associated with each live macrophage. Over a 5-day period, the numbers of live bacteria associated with macrophages decreased from 97 to 0% at an MOI of 100:1, while at an MOI of 500:1, live bacteria were still evident at day 4 in association with 11% of macrophages. Dead bacteria were apparent at both MOI and at all time points examined but as a small proportion (<10%) of the total population and principally where ≤5 bacteria were associated with each macrophage. However, as the macrophages were infected while subconfluent and continued to divide during the period of the assay, the percentage of macrophages with associated bacteria at day 1 cannot be equated with the percentage of macrophages with associated bacteria at day 4.

Transmission electron microscopy (TEM) of samples of cultured macrophages infected at an MOI of 500:1 at 4 h postinfection showed bacteria localized within vacuoles (Fig. 5a) in around 90% of the infected cells examined. The bacteria were present in low numbers in the phagosomes, usually singly, although two or three bacteria were occasionally observed within the same vacuole. No bacteria were observed free in the cytoplasm in the cell line. Infected P388D1 cells were also examined at 2 and 4 days postinfection. While bacteria were still found singly in phagosomes, they were present in only around 30% of the cells examined. TEM of peritoneal exudate cells from mice infected with *B. bronchiseptica* CBF2 showed the cells to be mainly PMN. Samples were harvested at 24 and 48 h postinfection from mice infected with 10^9 and 10^8 CFU of *B. bronchiseptica* CBF2. Bacteria were found in cells in all mice infected with 10^9 CFU and in no samples from mice infected with 10^8 CFU. Where present, bacteria were packed within individual vacuoles in high numbers (Fig. 5b and c) and, in around 5% of PMN, bacteria appeared to be free in the cytoplasm.

Macrophage viability after infection with *B. bronchiseptica*. To determine whether *B. bronchiseptica* CBF2 exerted any cytotoxic effects on macrophages, CellTiter 96 nonradioactive cell viability assays were carried out at MOI of 0:1, 1:1, 10:1, 100:1, 500:1, and 1,000:1 over a period of 4 days. No evidence for macrophage death in response to bacterial infection at any of the MOI used was found (Fig. 6 and data not shown). The percent viability of infected macrophages (MOI of 100:1) was also characterized by trypan blue exclusion and fluorescent staining (LIVE/DEAD *Bac*Light), and no significant differences between infected and noninfected cultures were observed (data not shown).

DISCUSSION

Although some early reports suggested that *B. pertussis* could be found intracellularly in HeLa and mouse kidney cell

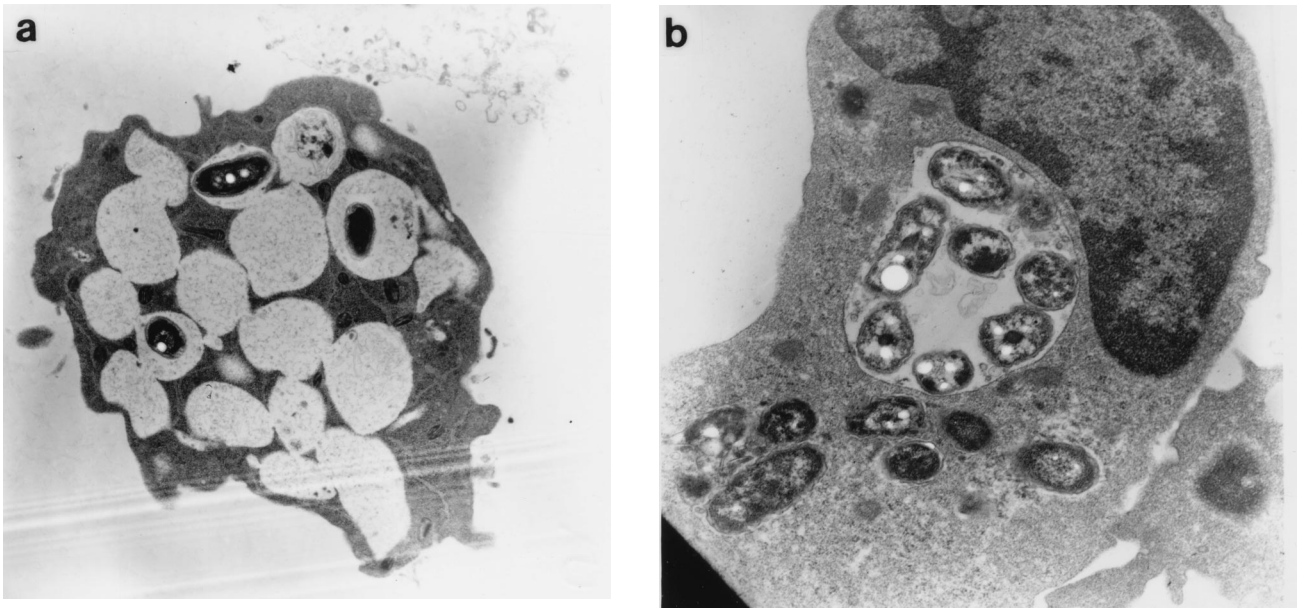

FIG. 5. (a) TEM demonstrating intracellular *B. bronchiseptica* CBF2 enclosed within phagosomes in culured macrophages (P388D1), infected at an MOI of 500:1, at 4 h postinfection. Magnification, ×14,000. (b) Peritoneal exudate cells from mice infected with ~10^9 CFU at 24 h postinfection. Bacteria are localized mainly in the phagosomal vesicles but are also evident in the cytoplasm. Magnification, ×24,000. (c) Peritoneal exudate cells from mice infected with ~10^9 CFU at 48 h postinfection. Magnification, ×14,000.

lines (9) and in murine macrophages (4), *Bordetella* species in general were not considered to live intracellularly until more recently, when it was established that *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* were able to be internalized by a variety of cultured eukaryotic cells. The importance of cell-mediated immunity in protection against experimental pertussis has been linked to a possible intracellular stage of infection (38). We have characterized the uptake and persistence of *B. bronchiseptica* in a murine macrophage-like cell line and in murine peritoneal cells by using a bioluminescence-based assay to monitor the fate of the bacteria. The advantages of using *lux* genes as a reporter have been widely described in studies relating to in vitro gene expression. To date, however, bioluminescence has not been exploited extensively in the study of mammalian host-microbe interactions, although some studies have used *lux*-tagged *Salmonella typhimurium*, *Mycobacterium tuberculosis*, and *Yersinia pseudotuberculosis* to evaluate infections in mice (7, 16, 24). These three studies showed that intracellular viability can be monitored directly, as the intensity of bioluminescence in aerobic bacteria is a reflection of metabolic activity and is dependent on a fully functional electron transport chain (34). Only one of these studies, that of *S. typhimurium* invasion in mice, used a construct containing the whole luciferase operon. This avoids the necessity of exogenous aldehyde addition, but, as the operon was carried on a plasmid, the requirement of positively selecting for maintenance of the *lux* reporter remained (7).

Mutagenesis of bordetellae with various Tn*5* derivatives has proved valuable for characterizing *bvg*-regulated gene expression (47, 48). In this study, *B. avium*, *B. parapertussis*, and *B. bronchiseptica* were successfully mutagenized with a suicide vector carrying the *lux* genes from *P. luminescens* on a mini-Tn*5* derivative. However, for *B. pertussis* 18-323, insertion mutants were not detected with either the parent vector pUTmini-Tn*5*km or its *lux* derivative, pUTmini-Tn*5*km*lux*. As pUTmini-Tn*5*km has been used to generate exconjugants in the *B. pertussis* strains Tohama I and SK73 (35), this may suggest that transposition of these plasmid vectors is strain dependent in *B. pertussis.*

The limit of detection of bioluminescent intracellular bacteria is determined by a number of factors, including the sensitivity of the luminometer, the numbers of bacteria internalized, and the amount of lumincescence emitted by the bacterium under investigation. In this study, a reporter strain expressing bioluminescence constitutively at high levels was used, as *B. bronchiseptica* is found within eukaryotic cells in relatively low numbers. At 4 h postinfection, internalized Lux^+ *B. bronchiseptica* organisms were found in murine macrophages as a constant proportion of the inoculum (~1%) at different MOI, as quantified by both luminometry and plate counts. This value is similar to that obtained in previous studies with *B. bronchi-*

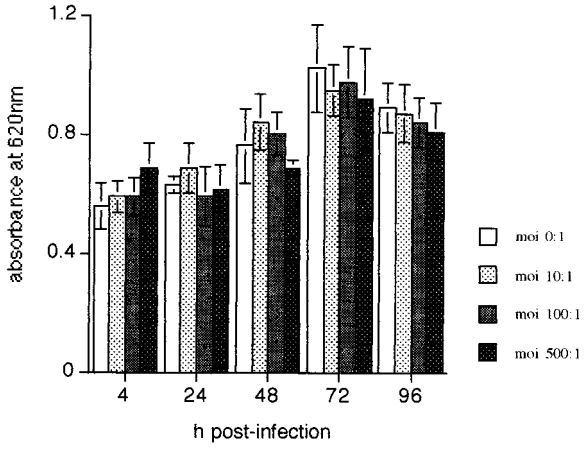

FIG. 6. Viability of cultured macrophages infected with *B. bronchiseptica* CBF2. PD3881 cells were infected with bacteria for 2 h at various MOI, and the cells were incubated with polymyxin for 90 min to kill extracellular bacteria. After a wash, viability (MTT) assays were carried out at intervals over a period of 4 days. Assays were carried out twice in triplicate. Bars indicate standard deviations of one assay.

septica, in which 0.1 to 5% of the total inocula were internalized into epithelial cell lines, dendritic cell lines, and macrophages (1, 20, 44). The reason for this low level of bacterial entry and survival is not clear, but it may indicate the presence of a subpopulation of *B. bronchiseptica* which either is able to efficiently enter the cells or is able to survive the killing processes of phagocytes. In this regard, it was of interest to note that the initial light output from infected macrophages activated by IFN-γ was greater than that emitted from nonactivated macrophages, possibly indicating a greater level of bacterial attachment to, or uptake by, IFN-γ-treated cells.

Our results clearly show that the light output recorded from infected eukaryotic cells after polymyxin treatment is emitted in the main from intracellular bacteria. Lysis of the mammalian cells with digitonin, which has no effect on the viability of *B. bronchiseptica*, produced a rapid decline in light output as the internalized bacteria were exposed to antibiotic in the surrounding medium and were killed. In addition, our studies with CD suggest that uptake of *B. bronchiseptica* by macrophages occurred primarily by microfilament-dependent phagocytosis, as has been reported previously for *B. bronchiseptica* in mouse dendritic cells (21) and for *B. pertussis* in macrophages (18). Inhibition of phagocytosis by CD reduced the light output and hence the internalization of *B. bronchiseptica* by macrophages to almost basal levels. Thus, the procedures for removal and killing of extracellular bacteria by washing and by treatment with polymyxin were very efficient, even in the presence of eukaryotic cells, and the presence of surviving extracellular bacteria contributed little, if at all, to the total light output recorded in the luminometry assays.

A decline with time in light output from infected cultured macrophages was evident in the luminometry assays, which was most marked at high MOI, and there are a number of possible explanations for this. Firstly, a small proportion of extracellular bacteria may have persisted within the macrophage monolayer in a protected extracellular microenvironment which, on disruption of the monolayer and exposure to polymyxin, were then killed. This would be of particular concern where low-level invasion or internalization is being assayed by using plate counts, as those bacteria designated as intracellular may in fact represent extracellular survivors. Secondly, it may represent an ongoing killing of internalized bacteria by macrophages. Thirdly, it may represent a decline in the viability of the macrophages which, in turn, would lead to entry of polymyxin and killing of the intracellular bacteria.

At an MOI of <500:1, viable numbers of intracellular *B. bronchiseptica* declined markedly over a 4-day assay period, while at an MOI of 500:1 or above, bacteria were recovered 4 days postinfection with only a slight reduction in bacterial numbers. In addition, light output was recorded from infected cultured macrophages at day 4 only when an MOI of ≥500:1 was used. This suggested that a critical number of bacteria were necessary for persistence within the cultured macrophages, as was reported for *B. pertussis* where, at an MOI of <100:1, they do not persist (18). The indication that persistence of *B. bronchiseptica* is dependent on the initial inoculum was reinforced by the observation that no light output was detected at 24 and 48 h postinfection in peritoneal exudates of mice injected intraperitoneally with 10^8 CFU but was detected when mice were infected with 10^9 CFU. TEM of peritoneal exudate cells recovered at both time points indicated that bacteria were found intracellularly only where mice were infected at the higher dose. The significance of this observation to events in vivo demands further investigation, as it would suggest that *B. bronchiseptica* persists intracellularly, at least in phagocytes, only once infection is well established. An alternative explanation is that the intracellular bacteria have not persisted within individual peritoneal cells for the 24- or 48-h time periods but may have more recently entered these cells. The relevance of MOI in internalization and survival of *B. bronchiseptica* in eukaryotic cells in vitro has not been examined previously, and the decline in numbers of viable intracellular bacteria observed in other studies may be attributable to the MOI used in the assays.

The mechanism by which *B. bronchiseptica* resists intracellular killing in both professional and nonprofessional phagocytes is unresolved. Intracellular *B. bronchiseptica* organisms have been shown to reside generally within phagosomes, although one report indicated that about 5% of intracellular bacteria were free in the cytoplasm at 2 to 3 days postinfection (44). In our study, *B. bronchiseptica* was evident only in low numbers and was contained in phagosomes in murine macrophages. In PMN, where bacteria were found in much higher numbers, some bacteria appeared to be present in the cytoplasm. If *B. bronchiseptica* is able to escape the phagosome and reside in the cytoplasm, it is at a much lower level than found with other intracellular bacteria such as *Shigella* and *Listeria* species, which are known to utilize this mechanism as a means of evading killing (19, 27). There was no evidence for intracellular replication of *B. bronchiseptica* in this study, either by plate counts, luminometry assays, or TEM, which is in agreement with previous reports (20, 44).

ACT, a *bvg*-regulated virulence factor in both *B. pertussis* and *B. bronchiseptica*, was initially described as protecting *B. pertussis* against killing by macrophages (32), but more recently it has been reported to induce apoptosis in macrophages (29). Similarly, *Salmonella* species were shown to be cytotoxic for macrophages (5), although in another report they have also been reported to survive and replicate within macrophages (3). In the present study, *B. bronchiseptica* appeared to be nontoxic for cultured macrophages over a 4-day period. As both *B. bronchiseptica* and *B. pertussis* produce ACT, the reason for the long-term persistence of the former but not the latter in vitro is intriguing and may be related to the observation that survival of *B. bronchiseptica* is *bvg* independent. Also, as persistence of *B. bronchiseptica bvg* mutants appears to be enhanced in eukaryotic cells (1, 44), this may suggest that down-regulation of

virulence factors facilitates intracellular persistence. However, it is not known which virulence factors, if any, are expressed intracellularly.

A criticism often made of assays carried out in tissue culture, especially where numbers of intracellular bacteria are relatively low and where no animal model exists to confirm in vitro findings, is that the test system may not be representative of the true in vivo situation. We have sought to address this issue both by activating macrophage cell lines and by comparing killing and survival of *B. bronchiseptica* directly in cultured cells and in freshly harvested phagocytes. Pretreatment of cultured macrophages with IFN-γ, which is known to activate the antibacterial mechanisms of murine macrophages via induction of nitric oxide (11), appeared to enhance the bactericidal abilities of the cells. When macrophages were pretreated with IFN-γ for 72 h preinfection, the light output was lower than that measured from infected but untreated macrophages. However, a significant proportion of the bacteria were still able to survive intracellularly. When *B. bronchiseptica* organisms were incubated both with freshly harvested mouse peritoneal exudate cells and with the nonactivated cell line, a remarkably similar pattern of internalization and persistence was evident, showing that the cell line did provide a representative model of intracellular survival for *B. bronchiseptica*.

Internalization and persistence of *B. bronchiseptica* in macrophages and in PMN, as demonstrated in this study, and in other eukaryotic cell lines, as reported elsewhere, clearly suggests that *B. bronchiseptica* may have an intracellular phase during the normal infectious process. Bioluminescence has proved to be an effective in vitro reporter. Its use has shown that *B. bronchiseptica* is internalized by professional phagocytes by a microfilament-dependent process in a dose-dependent manner and that the bacterium survives intracellularly and persists when a critical population size is present. In addition, preliminary in vivo studies have demonstrated that intracellular bioluminescent *B. bronchiseptica* can be recovered and detected from mice infected intraperitoneally. Further studies of the behavior and invasive properties of *B. bronchiseptica* in animal models of infection will be enhanced by the use of *lux*-tagged bacteria and will help elucidate the extent of the invasive properties of this bacterium in vivo.

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