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Phase Variation Affects Long-Term Survival of *Bordetella bronchiseptica* in Professional Phagocytes

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Several *Bordetella bronchiseptica* isolates were investigated for intracellular survival in macrophages. A significant number of viable bacteria of all strains could be recovered even after 96 h postinfection. In all cases *bvg* mutants of the *B. bronchiseptica* strains showed a significant survival advantage over the respective wild-type strains. The bacteria were already located in phagolysosomes early after uptake. Neither opsonization of the bacteria nor activation of the macrophages with gamma interferon or lipopolysaccharide prior to infection affected uptake and survival of the bacteria.

Bordetella bronchiseptica is very closely related to *B. pertussis*, the etiological agent of whooping cough (26). Whereas *B. pertussis* is an obligate human pathogen, *B. bronchiseptica* has a broader host range causing respiratory disease in several mammalian species but only occasionally in man (26, 28). Both organisms produce a series of highly related virulence factors such as adhesins and the adenylate cyclase toxin (26). However, only *B. pertussis* is able to produce the pertussis toxin interfering with regulatory G proteins in the cell membrane in eukaroytic cells (26). The expression of these factors is coordinately regulated by the BvgAS two-component system (3, 4, 26) in response to certain environmental stimuli, a phenomenon termed "phenotypic modulation." The *bvgAS* locus is genetically unstable, and spontaneous avirulent phase III variants occur, which do not produce these virulence factors due to mutations in the regulatory system (26). The in vivo relevance of phase variation and phenotypic modulation is not clear.

A series of recent investigations demonstrated that infection of mice with *B. pertussis* or *B. bronchiseptica* causes very significant Th1-type T-cell responses. As such Th1-type responses are generally associated with infections by intracellular pathogens, an intracellular localization during infection by *Bordetella* species was suggested (2, 10, 17–19). These immunological data are in line with results reported by several groups which showed that *B. pertussis* is indeed able to invade cultured epithelial cell lines but also primary epithelial cells (4, 6, 16). Furthermore, *B. pertussis* is able to persist in professional phagocytic cells with a significant survival rate at least for several hours (7, 20, 24, 25). Similarly, *B. bronchiseptica* is able to enter and to persist in epithelial cell lines (21, 22). However, in marked contrast to *B. pertussis*, *B. bronchiseptica* does not require *bvg* activated virulence genes for invasion in epithelial and dendritic cells (12, 21, 22). For both organisms, only very limited information is available about their intracellular destiny in phagocytic cells, although, after damaging of the epithelial cell layer, macrophages are encountered by these respiratory pathogens.

Therefore, the survival properties of several *Bordetella* strains, listed in Table 1, were analyzed in murine bone marrow-derived macrophages (BMMs) and in J774 macrophage-like cells (ATCC TIB-67). The bordetellae were cultivated as described elsewhere (4). Nonhemolytic spontaneous phase variants from *B. bronchiseptica* BB7306 and MUCOB4 were isolated, which did not produce the various virulence factors including adenylate cyclase toxin, filamentous hemagglutinin, and pertactin. These phase variants carried mutations in the *bvg* locus as determined by allelic exchange experiments with the wild-type *bvg* locus (data not shown). BMMs were prepared as described elsewhere (14). Briefly, the BMMs were isolated from female BALB/c mice and cultured in RPMI-Click medium (Biochrom) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 2 mM L-glutamine (GIBCO), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 0.05 mM 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (Fungizone) (2.5 μg/ml), and 20% supernatant of L929 fibroblasts (conditioned medium) as a source of granulocyte-macrophage colony-stimulating factor. Approximately 10^5 macrophages were seeded in 24-well tissue culture plates (Becton Dickinson) for the infection assays or on 13-mm cover slides located within 24-well tissue culture plates for electron microscopy studies and cultured with 5% CO_2 at 37°C. Between days 7 and 10 the macrophages were infected with the bacteria. The J774 cells were treated as described previously (23). Infection of the macrophages was carried out

TABLE 1. Bacterial strains used in this study

Strain	Description	Source and/or reference
B. bronchiseptica		
BB7865	Wild type, Str^r	IRIS,[a] Culture Collection University of Göteborg (22)
BB7866	BB7865 Δ*bvgS* Nal^r	IRIS (22)
BB7306-I	Wild type	IRIS Manchester University Collection of Bacteria
BB7306-III	BB7306 *bvg*	This study
MUCOB4-I	Wild type	IRIS, Manchester University Collection of Bacteria
MUCOB4-III	MUCOB4 *bvg*	This study
B. pertussis		
Tohama I	Wild type	26
BP347	Tohama I *bvgS*::Tn*5*	26
E. coli K-12 DH5α		Gibco BRL

[a] IRIS, Istituto Ricerche Immunobiologiche, Siena, Italy.

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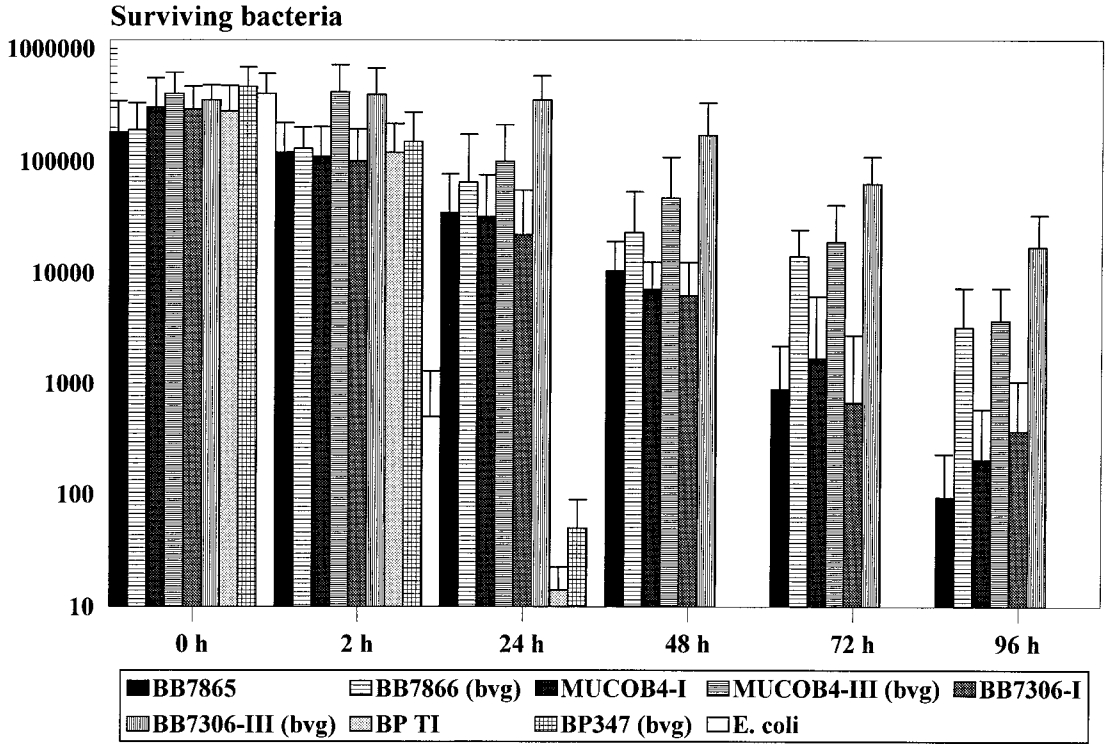

FIG. 1. Long-term intracellular survival in mouse BMMs of various *B. pertussis* and *B. bronchiseptica* wild-type and *bvg* mutant strains. The *B. bronchiseptica* and *B. pertussis* wild-type strains used are BB7865, MUCOB4-I, BB7306-I, and BP TI; the corresponding *bvg* mutants are BB7866, MUCOB4-III, BB7306-III, and BP347 (Table 1). The time scale refers to the time point of addition of gentamicin to kill extracellular bacteria. All experiments were carried out at least three times in duplicate, and the means of these data are presented.

at a multiplicity of infection of 100, as recently described (4, 14). Briefly, the bacteria were incubated with the cells for 1 h to allow their phagocytosis. A longer exposure of the bacteria caused cell death. Then, the extracellular bacteria were removed by extensive washing with phosphate-buffered saline, addition of 100 μg of gentamicin per ml, and further incubation for 2 h. For long-term survival experiments, the gentamicin concentration was then lowered to 10 μg/ml to minimize intracellular killing due to uptake of gentamicin by the macrophages (5). After lysis of the cells at various time points the viable counts were determined as described elsewhere (14, 22, 23).

About 2 to 5% of the bacteria were taken up by both types of macrophages, and no remarkable difference in the uptake efficiency between the wild type and the phase variants could be noted (data not shown). Two hours after infection no reduction of the number of viable *Bordetella* strains could be detected, whereas the *Escherichia coli* strain was killed very efficiently. Two days after infection no viable *B. pertussis* could be recovered from the cells, whereas all *B. bronchiseptica* strains were only slightly reduced in their viable counts (Fig. 1). Significant numbers of viable bacteria could be recovered even 96 h after infection. Interestingly, neither opsonization of the bacteria with 20% fresh mouse serum nor activation of the macrophages with 20 U of gamma interferon (Genzyme, Cambridge, Mass.) per ml or 100 ng of lipopolysaccharide per ml (derived from *E. coli* O26:B6; Sigma, Deisenhofen, Germany) (15) prior to infection had any influence on bacterial uptake or intracellular survival (data not shown). The activated status of the macrophages was verified by the determination of nitrite production, as described previously (15). The results obtained with the BMMs and the J774 cells were very similar, although the number of surviving bacteria was significantly higher in the J774 macrophages (data not shown).

Surprisingly, in all cases the phase III derivatives of the *B. bronchiseptica* isolates showed a significantly higher survival rate than the wild-type strains ($P < 0.01$). As the MIC of gentamicin is the same for the wild-type strains and their *bvg* derivatives (data not shown), this unexpected result is not due to a higher level of resistance against gentamicin of the phase III variants. To further confirm this surprising finding, we in-

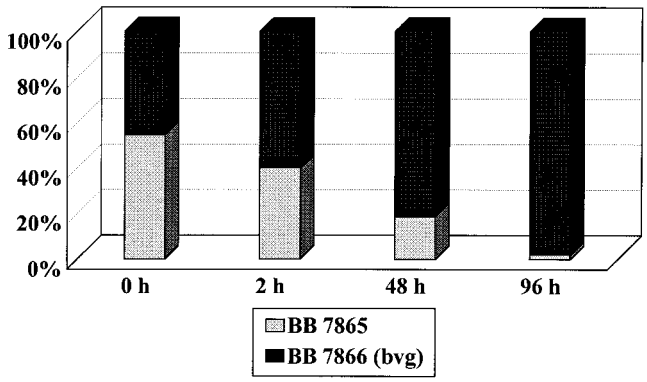

FIG. 2. Relative survival of wild-type (BB7865) and *bvg* mutant (BB7866) *B. bronchiseptica* strains after infection of J774 macrophages with a mixed suspension of the two strains. The time scale refers to the time point of addition of gentamicin to kill extracellular bacteria. The experiments were carried out three times in duplicate.

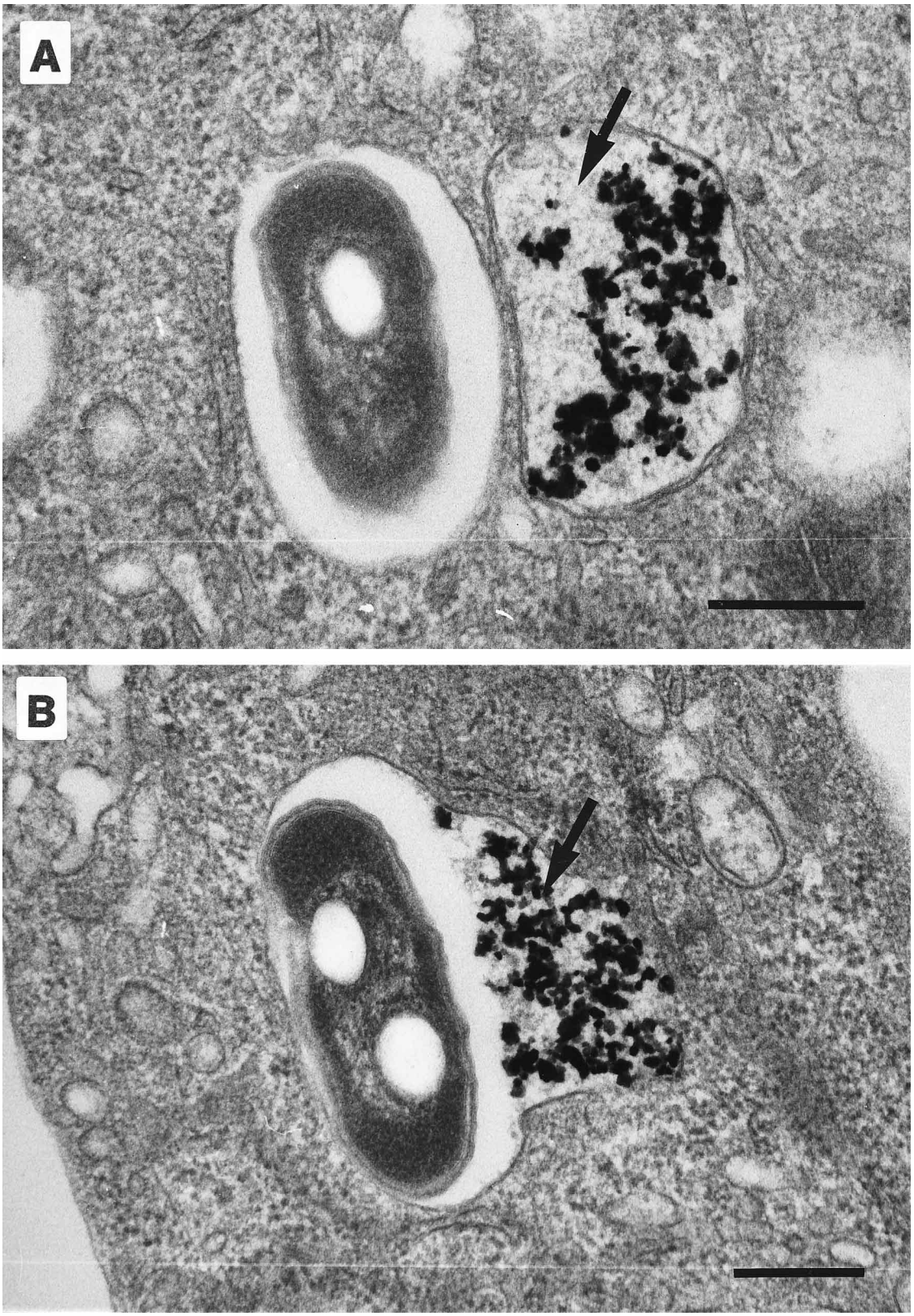

FIG. 3. Transmission electron micrographs (TEM) demonstrating intracellular *B. bronchiseptica* strains in mouse BMMs. Lysosomes were labelled with Thoria sol. (A) Unfused lysosome in the neighborhood of a phagosome containing a single *B. bronchiseptica* BB7866 cell; (B) lysosome fused with a phagosome about 2 h after infection. Bars, 0.5 μm. Arrow, Thoria sol-labelled lysosomes. Thoria sol labelling and TEM procedures have already been described (23).

oculated the BMMs and the J774 macrophages with mixed cultures of the wild-type BB7865 and phase III BB7866 strains. Again, as shown in Fig. 2 for the J774 cells, the *bvg* mutant BB7866 showed a much better survival rate than the wild type.

These results indicate that the production of *bvg* activated virulence genes may be of disadvantage for the intracellular bacteria. The adenylate cyclase toxin can induce apoptosis in phagocytic cells (13), and intracellular expression of this toxin is highly toxic for transfected cells (27). However, killing of their host cells may not be desired by the bacteria persisting in an intracellular niche. It is therefore likely that cytotoxic factors are downregulated in the intracellular milieu. To further prove this hypothesis, we investigated cytotoxic effects of the intracellular bacteria on their host cells. For this purpose, we determined the amount of lactate dehydrogenase (LDH) present in the culture supernatant as a marker for cell damage using the CytoTox 96 kit (Promega) (13). Approximately 10^4 BMMs were seeded in 96-well tissue culture plates and infected with bacteria followed by gentamicin treatment as described above. Alternatively, the macrophages and the bacteria were incubated for up to 8 h without addition of gentamicin. Percentage of cell damage was calculated according to the manufacturer's instructions. As expected, incubation of the cells with either *B. pertussis* or *B. bronchiseptica* wild-type strains caused massive liberation of LDH (about 40-fold the background level). No cytotoxic effects could be detected with the phase III derivatives of the two strains. In contrast, intracellular wild-type strains did not cause any liberation of LDH above the background level (data not shown). Accordingly, trypan blue staining of the cells confirmed the integrity of the macrophages throughout the long-term survival experiments, and no induction of apoptosis induced by the intracellular bacteria could be detected (data not shown). It is therefore likely that a downregulation of the adenylate cyclase toxin occurs in the intracellular compartment, reducing its activity to negligible amounts.

So far, very little is known about the mechanisms allowing these bacteria to resist the antimicrobial activities of the phagocytic cells. Recently, it was shown that early killing of *B. pertussis* and *B. bronchiseptica* in phagocytic cells does not occur, although their engulfment induces a significant oxidative burst activity (8, 24). However, evidence was provided that *B. pertussis* inhibits phagosome-lysosome fusions, thus preventing its exposure to lysosomal antimicrobial compounds (24, 25). To investigate phagosome-lysosome fusion events after engulfment of *B. bronchiseptica*, we labelled secondary lysosomes of BMMs with the electron-dense marker Thoria sol as recently described (23). In contrast to the results reported for *B. pertussis*, this analysis showed that shortly after engulfment of *B. bronchiseptica* by the macrophages fusion events between bacterium-containing phagosomes and lysosomes are occurring (Fig. 3). Indeed, after 1 day at least 50% of the *B. bronchiseptica*-containing vacuoles were fused with lysosomes, independently of the phase of the bacteria. With time, the number of fused vesicles further increased. A similar ratio of fusion events was also observed for the *E. coli* control strain (data not shown).

Therefore, although closely related, *B. pertussis* and *B. bronchiseptica* show several remarkable differences regarding their interactions with phagocytic cells. Whereas *bvg* activated factors including the adenylate cyclase toxin contribute to uptake and intracellular survival in the case of *B. pertussis* (7), the expression of these factors is not relevant or may even be harmful for *B. bronchiseptica*. In addition, *B. pertussis* is able to interfere with the fusion of lysosomes with bacterium-containing phagosomes (24, 25), whereas *B. bronchiseptica* is clearly entrapped in phagolysosomes early after engulfment. Nevertheless, *B. bronchiseptica* has a strong survival potential in professional phagocytes, although it is obviously attacked by reactive oxygen metabolites and lysosomal material. Reasons for the better survival in mouse macrophages of the phase III *B. bronchiseptica* strains compared to the respective wild-type strains are not clear. It is possible that either a low-level expression of cytotoxic factors by the wild-type strains, which interfere with signal transduction cascades of the host cells, may impair their intracellular survival potential, or alternatively, it may be that factors negatively regulated by the *bvg* system contribute to intracellular survival of *B. bronchiseptica* (1). The phagosomes of macrophages are therefore the first potential in vivo compartment in which phase III variants of *Bordetella* strains, which are generally considered to be avirulent, have a survival advantage over the phase I wild-type bacteria. The properties of phase III strains described in this report remind us of the recent description of chronic human infections in the respiratory tract caused by phase variants of *B. bronchiseptica*. After establishing the chronic infection, these bacteria had lost the ability to produce the adenylate cyclase toxin (11), which is believed to be essential for the initial colonization of the respiratory tract (9). It is tempting to speculate that the intracellular location may be a niche for persistent *B. bronchiseptica* and lack of expression of cytotoxins may improve the chance of bacterial persistence. The significant intracellular survival capacity of *B. bronchiseptica* in epithelial cells (23) and phagocytes may contribute to the strong cellular immune responses observed after respiratory infection with this organism (10).

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