Associated roles of hemolysin and p60 protein for the intracellular growth of *Bacillus subtilis*

Jarosław Wiśniewski, Agata Krawczyk-Balska & Jacek Bielecki

Department of Applied Microbiology, Faculty of Biology, Institute of Microbiology, University of Warsaw, Warsaw, Poland

Correspondence: Jacek Bielecki,
Department of Applied Microbiology, Faculty of Biology, Institute of Microbiology, University of Warsaw, 02-096 Warsaw, Str. Miecznikowa 1, Poland.
Tel.: +48 22 5541304;
e-mail: jbielecki@biol.uw.edu.pl

Received 17 May 2004; revised 15 October 2004; accepted 13 September 2005
First published online 21 February 2006.
doi:10.1111/j.1574-695X.2006.00029.x
Editor: Alex van Belkum

Keywords
*Listeria monocytogenes; Bacillus subtilis; iap gene; hemolysin.*

Abstract

Hemolysin expressing *Bacillus subtilis* strain (*B. subtilis* ble/hlA) was used as a carrier for listerial protein p60 to study the impact of this protein on bacterial virulence independent of other gene products of *Listeria monocytogenes*. *Bacillus subtilis* ble/hlyA exhibited longer cell chains than *B. subtilis* ble/hlyA/iap. Recombinant *Bacillus* strains are able to adhere to the mouse macrophage-like J774 and human epithelial-like Int407 cell lines. The bacterial number of *B. subtilis* ble/hlyA/iap strain that adhered to the Int407 cell lines was 2.52-fold higher, and its invasion level strain was 2.66-fold higher than that observed for the hemolytic strain. Microscopy analysis of infected monolayers showed that recombinant *B. subtilis* cells were localized inside the cytoplasm of epithelial cells, near to the nuclei, in cellular compartments with low internal pH. Furthermore, in cells infected with bacteria, the actin structures rapidly changed and accumulation of a fat, wide actin layer around the nucleus zone was observed.

Introduction

*Listeria monocytogenes* is a facultative intracellular pathogen mainly causing meningitis and septicemia in humans and animals (Gray & Killinger, 1966; Farber & Peterkin, 1991). *Listeria* invasion does not appear to be restricted to M cells and progresses through epithelial cells above Peyer’s patches (Pron et al., 1998; Daniels et al., 2000). After *L. monocytogenes* is phagocytosed by a macrophage, it dissolves the phagosomal membrane via listeriolysin (LLO, HlyA) activity and enters the cytoplasm.

All species of the genus *Listeria* secrete a major extracellular protein called p60, or Iap (invasion associated protein). In bacteria grown to the early stationary phase, about 25% of the extracellular protein p60 was estimated to be associated with the cell surface (Ruhland et al., 1993). The roles of Iap in pathogen survival *in vivo* and host cell invasion *in vitro* have been studied in several laboratories.

Intracellular bacteria and viruses are controlled by T-cell-mediated immune mechanisms. Satisfactory control of these pathogens would be best achieved by vaccines that efficiently stimulate protective T cells. A general strategy for development of vaccines against intracellular pathogens includes the following: (i) production of the antigen in the host cell; (ii) antigen degradation (antigen processing); and (iii) the presence of appropriate amino acid motifs in the protein sequence for allele-specific MHC class I presentation.

Iap protein is processed by the host cell into the nonamer peptide p60 217–225 and presented to cytotoxic T lymphocytes by the H-2Kd MHC class I molecule. Quantitation of p60 217–225 in infected cells shows that this epitope is detectable within 2 h of infection and, after 9 h infection, there are over 3000 epitopes per infected cell. This contrasts with listeriolysin 91–99, the other major *L. monocytogenes* epitope, which is present in quantities below 200 epitopes per cell until 5 h of infection and reaches 800 epitopes per cell 9 h after infection. Synthetic peptides derived from the variable region of the *L. monocytogenes* p60 protein may be useful for the development of an immunological diagnostic assay (Bubert et al., 1994). (Harty & Pamer, 1995) showed that H-2d restricted CD8 T-cells with specificity for the secreted protein p60 can protect against infection. These authors hypothesized that secreted bacterial proteins are the most important targets for protective CD8 T-cell-mediated immunity.
Studies on attenuated strains of intracellular pathogens aimed at their use as carriers of antigens secreted inside the cytoplasm have been initiated in several laboratories. There are a very limited number of bacterial vectors with acceptable safety and performance profiles that would warrant their use in humans. Naturally occurring attenuated mutants of *L. monocytogenes* may be used as vaccine candidates for listeriosis and as carriers, but knowledge regarding listerial pathogenesis, understanding of the listerial biological cycle and the availability of genetic tools is not yet sufficient for the use of these strains.

The experiments described here show that the soil bacterium *B. subtilis* secretes determinants of pathogenesis of *L. monocytogenes*, which can be used as a prototype for an intracytoplasmically growing bacterium, triggering a mainly immune response, making it seem an ideal alternative carrier.

**Materials and methods**

**Bacterial strains and growth conditions (Table 1)**

*Bacillus subtilis* was grown on brain heart infusion agar (BHI, Difco Laboratories,) or in BHI broth at 37°C. Stock cultures were maintained as cell suspensions at ~70°C in brain/heart infusion broth containing 50% (volume in volume, v/v) glycerol. For intracellular growth assay, cultures were grown in 10 mL BHI in a 250 mL flask with vigorous shaking at 37°C. *Bacillus* cells were washed twice with PBS (pH 7.4) and 20μL of this suspension was used immediately to infect monolayers. All growth media and solutions used in these procedures were supplemented with 10 mM IPTG.

**Tissue culture cells and growth medium (Table 1)**

The mouse macrophage-like cell line J774 was grown in 10 mL Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS) decomplemented for 30 min at 56°C, 100 units of penicillin per mL, 10 μg streptomycin per mL and 2 mM l-glutamine (5% FCS/DMEM) in 75 mL flasks. The epithelial intestine cell line Int407 (ECACC 85051004) was grown in 10 mL of 5% FCS/DMEM and fed twice a week with 10 mL of medium. The cells were kept in a humidified incubator at 37°C and supplied with a mixture of 6% CO₂–94% air.

**Fluorescence procedures**

Int407 cells were washed three times with PBS (pH 7.4), and fixed with 3% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature. Cells were opened for internal staining by treating them for 3–5 min with 0.1% Triton® X-100 in PBS (pH 7.4). F-actin staining with phallotoxin conjugated Alexa Fluor 488 was performed under the conditions recommended by the producer (Molecular Probes, Eugene, OR). Labeling of cell acid compartments was performed by incubating the coverslip with established cultures of Int407 cells in 5% FCS/DMEM supplemented with acridine orange (5 μg mL⁻¹). Preparations were washed, mounted, observed live with epifluorescence, and recorded with a digital camera. *Bacillus subtilis* strains were labeled with fluorescein isothiocyanate as described previously (Wadsworth & Goldfine, 1999) with the following modifications: bacteria were grown overnight at 37°C with shaking, the culture was diluted 1:10 with fresh medium and grown for 3 h at 37°C with aeration, 1 mL was removed, washed, stained and used to infect cells.

**Adherence assay**

After incubation with bacteria for 30 min, the cells were washed and lysed. The percentage of cell-associated bacteria was calculated as follows: [(number of cell-associated bacteria)/(total number of bacteria added) × 100]. Alternately, fluorescein isothiocyanate-labeled *B. subtilis* cells were used to infect J774 cells. At 10 min postinfection, cells were washed, and stained with 10 μg mL⁻¹ ethidium bromide. Cells were counted with the 590 nm filter, and bacteria were viewed with a 520 nm fluorescein filter. For each time point, 150–200 J774 cells with associated bacteria present in the field of view (760.9 μm per 570.7 μm) were viewed with a fluorescence microscope. Experiments were repeated at least three times.

**Intracellular growth assay**

Cultures of Int407 or J774 cells were established by adding 1 × 10⁶ cells in 5 mL of 5% FCS/DMEM with 10 mM IPTG to 60 mm petri dishes with round coverslips (12 × 1 mm; Propper Manufacturing). After overnight (J774) or 48 h...
incubation (Int407) at 37 °C under 6% CO₂, 20 μL B. subtilis cells was added per dish, and the dishes were incubated for an additional 60 min. Cultured cells were washed and 5 mL of warm 5% FCS/DMEM added to each dish. After 30 min the culture supernatant was removed and 5 mL of warm medium supplemented with gentamicin sulfate to 100 μg mL⁻¹ was added to each dish to inhibit extracellular growth of bacterial strains. Extracellular bacteria are killed by this antibiotic treatment but the viability of intracellular bacteria is not affected (Stoiber et al., 2001). Following in vitro infection for 2, 5 and 8 h, the bacterial numbers were measured by lysing the remaining cells after vigorously agitating the coverslip in 5 mL of water (1774) or 0.1% Triton X-100 in sterile water (Int407) in 15 mL conical tubes, and plating dilutions of the lysate on BHI agar plates. The data presented in this work represent the averages of the number of bacteria from three coverslips. In experiments designed to evaluate the role of vacuolar pH, chlorquinoline (7-chloro-4-[diethylamino-1 methyl butylamino]-quino-line) obtained from Sigma Chemical (St Louis, MO), was added to the growth medium 2 h after infection.

**DNA hybridization procedures**

For colony hybridization the rapid procedure for colony screening described by (Forster et al., 1990) was used. Digested DNA was transferred to nylon filters using the method described by Rosenshine & Finlay (1993). The filters were hybridized with DNA probes that had been labeled in vitro with the DIG Labelling System (Boehringer Mannheim) under the exact conditions recommended by the supplier. Hybridizations were performed at 68 °C under conditions of high stringency. Visualization with the DIG Nucleic Acid Detection System was performed as recommended in the manufacturer’s instructions.

**Transfer of plasmids**

Plasmids were transformed into *Escherichia coli* strain MC 1061 and of B. subtilis by electroporation under conditions recommended by the supplier of the electroporator (Bio-Rad, Hercules, CA).

**Plasmid screening**

Plasmid DNA was isolated by the alkaline lysis technique of Birnboim & Dolly (1979) purified in CsCl-ethidium bromide density gradient as described by (Sambrook et al., 1989). Restriction endonucleases were used under the conditions recommended by the supplier (Gibco BRL, Gaithersburg, MD).

**Western blot analysis of p60 and LLO production by recombinant Bacillus subtilis strains**

Collected culture supernatants, after dodecyl sulfate polyacrylamide gel electrophoresis, were transferred to a nitrocellulose membrane. Proteins p60 and LLO were detected with a rabbit polyclonal antisera against *L. monocytogenes* 10403S (Kucharczyk TE, Warsaw, Poland). The blot was developed with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin.

**Visualization and analysis of results**

For visualization, coverslips were stained with Diff-Quik (American Scientific Products) and mounted in Permount mounting medium (Fisher Scientific). Microscopic observation and microphotographs were performed on Olympus IX70 microscope ImageMaster® VDS (Video Documentation System) by Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). Gels were analyzed by ImageMaster® 1D Elite v. 3.01 gel analysis software produced by NonLinear Dynamics (Newcastle upon Tyne, UK). Microphotographs were projected, visualized and analyzed with DP-SOFT (analySIS®) software produced by Soft Imaging Systems for Olympus, and the final montage was created with Adobe Photoshop, version 6.0. The lengths of the cells were determined by image analysis software. Twenty cells were measured per sample to investigate differences in bacterial lengths.

**Results**

**Construction of strain Bacillus subtilis ble/hlyA/ iap expressing hemolysin and p60 protein from Listeria monocytogenes**

Fragments of the chromosome of *Listeria monocytogenes* 1043S were cloned into pAG58 ble/hlyA plasmid vector as previously described (Bielecki et al., 1990). The first stage of the cloning procedure involved the construction of a derivative of plasmid pLIV containing fragments of the *L. monocytogenes* chromosome. DIG–nonradioactive colony hybridization reaction was used to localize the strain carrying the chromosomal iap sequence, and plasmid pLIV 900 carrying iap was localized. Xhol/BamHI 4.2-kb fragment with iap sequence was blunted with Klenow subunit I enzyme, ligated with EcoRV cut and dephosphorylated plasmid pAG58 ble/hlyA. The resulting plasmid, designated pAG58 ble/hlyA/iap (Fig. 1), was electro-transformed into B. subtilis strain with selection for cmR and bleR. As the result of this experiment 10⁴ transformants mL⁻¹ were obtained. Homologous recombination between the sequence DNA from the pAG58 ble/hlyA/iap and the B. subtilis
chromosomal sequences was expected to result in simultaneous incorporation of the plasmid into the bacterial chromosome (Youngman et al., 1989). Western blot analysis (Fig. 2, lane 4) showed a large amount of 60 and 58 kDa proteins released to the culture supernatant by the \( B. \) subtilis \( \text{ble/hlyA/iap} \) strain. Both proteins 60 and LLO present in culture supernatant from \( B. \) subtilis \( \text{ble/hlyA/iap} \) and \( B. \) subtilis \( \text{wt} \) (2), \( B. \) subtilis \( \text{iap} \) (3) and from \( B. \) subtilis \( \text{ble/hlyA/iap} \) (4) were detected with a rabbit polyclonal antiserum against total culture protein \( L. \) monocytogenes 10403S. The position of p60 and LLO are indicated on the right, and the position of molecular mass standards separated on the same gel on the left.

![Fig. 1. Construction of pAG58 ble/hlyA and pAG58 ble/hlyA/iap vectors carrying the listerial listeriolysin (hlyA) and p60 protein (iap) under the control of the \( P_{\text{spac}} \) promoter. pBR, sequences derived from the pBR322; \( Tn \), sequences derived from Trn917; SP\( \beta \), prophage of \( B. \) subtilis temperature phase \( SP\beta_{2} \); \( Tn \), prophage of \( B. \) subtilis temperature phase \( Tn_{917} \); cat, \( Cm \) acetyltransferase gene originally from pC194; ble, bleomycin resistance gene; lacO, repressor binding sequences from \( E. \) coli lac operator region; lacI, promoterless copy of \( E. \) coli lac repressor gene; \( P_{\text{pcn}} \), constitutive promoter derived from \( B. \) subtilis \( \text{licheniformis} \) penicillinase gene; \( P_{\text{spac}} \), strong promoter derived from \( B. \) subtilis phage SPO1 whose expression is constitutive except when lac repressor is bound to lacO; solid arrow, constitutive transcription directed by \( P_{\text{pcn}} \); arrow with broken line, IPTG-inducible transcription directed by \( P_{\text{spac}} \).](image1)

**Bacterial invasion and intracellular multiplication**

Adhesion of bacterial pathogens to the host cell is essential for internalization. To examine the interaction between \( B. \) subtilis strains and mammalian cells, Int407 culture monolayer was tested for susceptibility to bacterial adherence. The number of cell-associated bacteria, both attached and intracellular, was determined after 30 min of incubation of the cells with the bacteria (Fig. 3a).

Marked differences were observed in the adherence efficiencies of the \( B. \) subtilis strains to the human Int407 cells. \( B. \) subtilis expressing LLO was able to adhere to the mammalian cell line with 0.25% efficiency. The number of bacteria of strain \( B. \) subtilis \( \text{ble/hlyA/iap} \) secreting both LLO and protein p60 that adhered to the Int407 cell lines (0.63 ± 0.13) was significantly (2.52 times) higher for the hemolytic strain. The adhesion of \( B. \) subtilis recombinant strains with J774 cells was determined by the fluorescent method after 10 min of postinfection (Fig. 3b). The \( B. \) subtilis \( \text{ble/hlyA/iap} \) strain demonstrated higher levels of adherence (determined as the number of bacteria that fluoresced green, labeled with fluorescein isothiocyanate, per J774 cell) than the \( \text{ble/hlyA} \) strain (0.175 ± 0.0308 compared to 0.089 ± 0.0216, respectively). In *vitro* experiments showed that p60 promotes the adherence of \( B. \) subtilis to the surface of both Int407 and J774 cell lines.

The recombinant \( B. \) subtilis strain escapes the phagosome by hemolysin-mediated lysis of the phagosomal membrane and then survives in the cell cytosol. Microscopic observations of Diff-Quik-stained infected monolayers showed that \( B. \) subtilis cells were localized inside the cytoplasm of the
epithelial cells (Fig. 4a). In this experiment extracellular multiplication was prevented by the addition of 100 μg mL⁻¹ gentamicin. Bacteria present in the cytoplasm were localized close to the nuclei or in the form of microcolonies in the infected cell. When infected Int407 cells were incubated with bacteria under identical conditions without IPTG, no bacterial activity was observed (data not shown). Microscopic observation of infected tissue showed that after translocation to the interior of the cell, the recombinant Bacillus cells are able to replicate inside the cytoplasm (Fig. 4b). The parental B. subtilis strain did not invade the cells. The number of B. subtilis ble/hlyA/iap bacteria that entered the Int407 cell line was significantly higher than the number of B. subtilis ble/hlyA bacteria. The invasion level of B. subtilis ble/hlyA/iap was 2.66-fold higher for the hemolytic strain, but the generation times of both strains were comparable. In Int407 epithelial cells the number of gentamicin-resistant B. subtilis cells recovered from coverslips increased to 2.1 × 10⁴ (ble/hlyA) and 5.46 × 10⁴ (ble/hlyA/iap) after 7-h incubation.

**Bacterial morphology**

Microscopic examination of infected monolayer showed differences in the morphology of recombinant Bacillus strains (Fig. 5). The length of cells of B. subtilis strains ble/
hlyA and ble/hlyA/iap derived from several experiments were analyzed. The lengths of the cells were determined by image analysis with an Olympus IX70 microscope that was connected via an Olympus C5050 digital camera to DP-SOFT software. Twenty cells were measured per sample to investigate differences in bacterial lengths (Fig. 6). The hemolytic strain exhibited longer cell chains (8.28 ± 1.34 μm [mean ± standard error of the mean, SEM]) than the strain expressing hemolysin and p60 protein (4.33 ± 0.66 μm). Similar results were observed in liquid cultures of both bacterial strains (data not shown).

**Cytoskeleton rearrangements**

Epithelial cell line Int40 grown on rounded coverslips was infected with 10 μL B. subtilis ble/hlyA cells washed in PBS (pH 7.4). After 45 min incubation, cells were washed five times in PBS (pH 7.4) and fixed. Actin cytoskeleton was stained with phalloidin conjugated with Alexa Fluor 488. The results of this experiment are shown in Fig. 7. The Bacillus strain expressing listerial hemolysin caused cytoskeletal reorganization in Int407 cells (Fig. 7b). An accumulation of a fat, wide actin layer around the nucleus zone was observed, with bacteria often localized to this area in the cytoplasm. When incubated in media without IPTG, B. subtilis ble/hlyA strain cannot induce actin filament rearrangement (data not shown).

Acridine orange is one of the most popular and versatile fluorescent stains for histochemistry and cytochemistry and can provide a wide variety of information about the in situ content, molecular structure, conformation and environment of many nucleic acid-containing cell constituents (Darzynkiewicz & Kapuscinski, 1990). Inside Int407 cells, B. subtilis was located close to the (near) nucleus. Live cells that stained acridine orange, weakly basic amine, selectively accumulated in cellular compartments with low internal pH, indicating that this region of the cell is an acid compartment (Fig. 8).

**Effect of pH intravacuolar compartment on intracellular growth**

Ammonium chloride was used to investigate the role of acidification of the intravacuolar compartment on the intracellular growth of Bacillus subtilis ble/hlyA and ble/hlyA/iap cells (Fig. 9). When ammonium chloride was added to the growth medium 2 h after bacterial infection, the number of gentamicin-resistant cells of both Bacillus strains decreased. After 3 h incubation of the infected monolayers in medium with this compound we isolated only 5% viable B. subtilis ble/hlyA/iap and only 0.61% viable cells of hemolytic strain.
The effect of chlorquinoline, another lysosomotropic agent, on intracellular multiplication of both Bacillus strains was examined. The substance was added 2 h postinfection. Fresh medium with 100 μg mL⁻¹ chlorquinoline was added to the dishes and the number of gentamicin-resistant bacteria was measured after 3 h incubation (Fig. 9). We observed a decrease in the number of viable intracellular bacteria (defined by gentamicin resistance) isolated from infected monolayers; ble/hlyA/iap and ble/hlyA strains decreased to 8.57% and 0.26%.

Discussion

Listeriolyisin has been implicated as a key molecule in Listeria monocytogenes infection and is required for the lysis of phagosomal membranes (Mengaud et al., 1988; Portnoy et al., 1988, 1992; Cossart et al., 1989; Bielecki et al., 1990). This protein also appears to have a major role in initial interactions of L. monocytogenes with cells (Bielecki et al., 1990). Although the reason for this is not immediately obvious, recent data have supported multiple effects of listeriolyisin on eukaryotic cells. Listeriolyisin appears to be responsible for induction of mitogen-activated protein kinase prior to bacterial entry, and expression of listeriolyisin in eukaryotic cells leads to a strong mitogenic response (Kugler et al., 1997; Tang et al., 1998; Stoiber et al., 2001). Hence, listeriolyisin may be activating cellular pathways for uptake by modulating key enzymes in signal transduction.

Intestinal epithelial cell line Int407 was used as a model for investigation of cooperation and influence of HlyA and protein p60 on pathogenesis. In the present experiments, Bacillus subtilis ble/hlyA strain was used as carrier for protein p60 to study the impact of this protein on bacterial virulence independent of other gene products of L. monocytogenes. The experiments described here show that B. subtilis expressed protein HlyA is able to enter the intestinal epithelium cell line Int407. This suggests that the listeriolyisin protein, when secreted by B. subtilis, can disrupt the membrane of these cells, allowing the bacteria to replicate inside the cytoplasm. Expression of HlyA consequently promotes infection, and contributes to the ability of B. subtilis to invade Int407 cells. Our results demonstrate that protein p60 promotes invasion of B. subtilis into mammalian cells. Fluorescence labeling of bacteria is useful for studying short time physiologic J774 macrophage-like cell: bacteria interactions. The data show that the L. monocytogenes surface-associated proteins, p60 and internalin, act in concert to achieve optimal uptake into mammalian cells. These results confirm recent observations of (Hess et al., 1997), who used Salmonella typhimurium SL7207 as a carrier for secreted p60-HlyA fusion protein. In vitro experiments showed that p60 promotes invasion of recombinant Salmonella strain into hepatocytes and resting macrophages independent of complement. Uptake of wild-type L. monocytogenes EGD and L. monocytogenes BUG 8, an internalin-deficient strain, into hepatocytes was partially blocked by anti-p60 antibodies.

As previously reported from several laboratories, rough mutants of L. monocytogenes exhibit a similar
phenotype, with bacterial cells forming long cell chains. Addition of growth medium of wild strain or partially purified p60 protein caused disruption and disaggregation of cell chains to normal-sized single bacterial cells whose invasiveness was restored (Kuhn & Goebel, 1989; Bubert et al., 1994, 1997). Thus, for these cells, cell-free p60 not only causes decay of cell chains but actively participates in the invasion process.

Other researchers have shown that p60 possesses a murein hydrolase activity which is required for a late step in cell division (Wuenscher et al., 1993; Bubert et al., 1997). Cell-chain-disruption activity can be blocked by inhibition of the single cysteine residue that occurs in all p60 proteins at the same position in the C terminus (Wuenscher et al., 1993). The p60 protein shows sequence similarity with the repeat domain of an autolysin of Streptococcus faecalis and causes lysis of Micrococcus luteus. The results of an experiment in which hemolytic Bacillus strain exhibited longer cell chains than the strain expressing hemolysin and biological active p60 protein suggest that p60 acts as a murein hydrolase. This observation is in accordance with previous work which indicated that expression of the iap gene of L. monocytogenes in the L. monocytogenes rough mutant RIII and in B. subtilis DB104 caused the disruption of the cell chains which these two strains normally form under exponential growth conditions (Wuenscher et al., 1993).

To analyze the role of acidification of the vacuolar compartment in the infection process caused by B. subtilis ble/hlyA and B. subtilis ble/hlyA/iap we used ammonium chloride and chlorquinoline (Mestecky, 1987). Using weak bases, we can raise the pH of the acid intravesicular compartment to test the effect of an elevated intravesicular pH on intracellular surviving Bacillus strains. Chlorquinoline induced the appearance of numerous clear vacuoles in the cytoplasm. The vacuoles, very similar to those described previously (Fedorko et al., 1968; Wibo & Poole, 1972), developed in the perinuclear region and by 1–2 h had filled the cytoplasm of most cells. After incubation of Int407 cells in media with chlorquinoline or ammonium chloride we observed changes in actin cytoskeleton organization (data not shown). The concentration of chloroquine could seriously affect the actin filaments, decreasing the amount of long actin filaments. Because the internalization process is the result of a remarkable interaction between the bacteria and the host cell, destabilization of the actin cytoskeleton decreases the amount of viable intracellular bacteria. Changes in the acid compartment of Int407 cells caused by chlorquinoline and ammonium chloride decreased the viability of Bacillus strain inside the cell. Inside Int407 cells, B. subtilis is located close to the (near) nucleus. Live cells that stained acridine orange, weakly basic amine, selectively accumulate in cellular compartments with low internal pH, indicating that this region of cell is the acid compartment. Recent observations of (Li et al., 2000) showed that exposure of the cells to chloroquine increases the lysosomal pH from 4.8 to 6.5 and this effect is concentration-dependent. Acidification seems to be required for bacterial escape from the phagosome compartment, and for optimal activity of listeriolysin (Portnoy et al., 1992). On other hand, expression of the iap gene from the construct in the B. subtilis strain showed the following: (i) disruption of the cell chains which this strain normally forms under exponential growth conditions increases the amount of CFU; (ii) surface-associated protein p60 may act to achieve optimal uptake into host cells; (iii) protein p60 plays a role in listeriolysin-mediated hemolytic activity.

Actin organization is mediated by several actin-binding proteins under the control of various upstream signals ranging from phospholipids, Ca2+, to the Rho family GTPases, all of which show a direct response to pathogen-mediated stress (Rosenshine & Finlay, 1993). A distinct response to intracellular bacteria stress is the transformation of the Int407 actin cytoskeleton, resulting in the concentration of F-actin bundles in the region of the nucleus. Although the reorganization of the actin cytoskeleton is well documented, mechanistically we do not fully understand why the actin polymerized in this region of cytoplasm.

Intracellular bacteria are controlled by T-cell-mediated immune mechanisms. Satisfactory control of these pathogens would be best achieved by vaccines which efficiently stimulate protective T cells. Secreted bacterial proteins are the most important targets for protective CD8 T-cell-mediated immunity. Interestingly, studies have shown that particulate antigens, especially when presented as viable organisms, are more effective than soluble antigens in inducing local and generalized secretory and systemic immune responses (Mestecky, 1987; McGhee & Mestecky, 1990). At least three reasons can be given for this. First, the size and composition of particulate antigens may allow them to survive more effectively in the environment of the gastrointestinal tract. Second, some particulates are more efficiently absorbed by the gut, specifically through the M (microfold) cells into the Peyer’s patches. Third, soluble antigens cross the epithelial barrier of the gut in the form of low molecular weight peptides. The recognition of these peptides by lymphoid cells at sites other than in the Peyer’s patches has been proposed as the stimulus that initiates systemic tolerance after antigen feeding (Bland & Warren, 1986) and may provide a negative signal to the mucosal immune system.

The system of B. subtilis live vaccine-secreted determinants of pathogenesis L. monocytogenes may have future use as a carrier of foreign antigens. These antigens would be produced inside the cytoplasm of mammalian cells and after antigen degradation (antigen processing) of appropriate
amino acid motifs could be presented in the protein sequence for allele-specific MHC class I.

A distinct advantage of live oral vaccines is their capacity to persist in the host for a significantly long period and to induce both humoral and cellular local immune responses. Protection against tumors and many intracellular pathogens requires an effective class I-restricted cytotoxic immune response, which necessitates cytoplasmic localization of antigens. As a prototype of a newly constructed intracytoplasmically growing bacterium that triggers an immune response, B. subtilis expressing L. monocytogenes genes seems an ideal alternative carrier.

The B. subtilis strains expressing the cloned protein or recombinant protein in the cytosol were able to trigger efficient proliferation of the T-cell clones at equal efficiencies or higher than antigen presenting cells pulsed with the corresponding peptides.

It is necessary to stress that one of the aspects usually underestimated during the development of attenuated vaccine candidates is that of biosafety. There are a very limited number of bacterial vectors with acceptable safety and performance profiles that would warrant their use in humans. In the future, B. subtilis strains expressing listerial proteins and foreign antigen will be used as vaccine candidates for listeriosis and as carriers for foreign genes because there is not yet sufficient knowledge of listerial pathogenesis and the listerial biological cycle, or available genetic tools to use naturally occurring attenuated mutants of L. monocytogenes. It is essential to limit the possibility of the engineered microorganism to survive in the environment and reduce the possibility of lateral spread of a phenotype, which might result in the acquisition of virulence by nonpathogenic bacterial species.

Acknowledgement

This study was supported by the grant 4 P05A 067 11 from KBN (Poland).

References


