# Microinjection and growth of bacteria in the cytosol of mammalian host cells

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**Most facultative intracellular bacteria replicate in specialized phagosomes after being taken up by mammalian cells. Relatively few intracellular bacteria escape the phagosomal compartment with the help of cytolytic (pore-forming) proteins and replicate in the host cell cytosol. Without such toxins, intracellular bacteria cannot reach this cellular compartment. To circumvent the requirement of an "escape" step, we developed a procedure allowing the efficient direct injection of bacteria into the cytosol of mammalian cells. With this technique, we show that most bacteria, including extracellular bacteria and intracellular pathogens that normally reside in a vacuole, are unable to replicate in the cytosol of the mammalian cells. In contrast, microorganisms that replicate in the cytosol, such as *Listeria monocytogenes*, *Shigella flexneri*, and, to some extent, enteroinvasive *Escherichia coli*, are able to multiply in this cellular compartment after microinjection. Further *L. monocytogenes* with deletion in its PrfA-regulated *hpt* gene was found to be impaired in replication when injected into the cytosol. Complementation of the *hpt* mutation with a plasmid carrying the wild-type *hpt* gene restored the replication ability in the cytosol. These data indicate that cytosolic intracellular pathogens have evolved specific mechanisms to grow in this compartment of mammalian cells.**

Many pathogenic bacteria are able to trigger their uptake by mammalian cells, which is followed by efficient multiplication of the internalized bacteria inside of the host cells. Internalization of these bacteria involves normal phagocytosis when the host cells are professional phagocytes, e.g., macrophages, or triggered phagocytosis in the case of nonprofessional phagocytic host cells, such as epithelial cells, hepatocytes, fibroblasts, and endothelial cells (1, 2). After internalization, most intracellular bacteria reside and replicate inside membrane-bound vacuoles that are specifically modified by the different bacteria (3, 4). *Salmonella enterica*, *Legionella pneumophila*, members of the *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, *Brucella* spp., *Chlamydia*, *Rhodococcus equi*, and several others belong to this group of intracellular bacteria. A smaller group of intracellular bacteria, including *Shigella* spp., the closely related enteroinvasive *Escherichia coli* (EIEC), *Listeria monocytogenes*, *Listeria ivanovii*, and *Ricksettia* spp., can escape from the primary phagosome into the host cell cytosol where the bacteria proficiently replicate. These latter bacteria synthesize specific proteins that disrupt the phagosomal membrane, thus allowing bacterial entry into the cytosol. In *L. monocytogenes*, the required proteins are best characterized and comprise the pore-forming lysteriolysin (LLO) and two phospholipases C, PlcA and PlcB (5, 6).

It has been reported that the introduction and expression of the listerial *hly* gene (encoding LLO) in *Bacillus subtilis* leads to the release of these avirulent bacteria into the cytosol of mammalian cells where they apparently replicate (7). This finding raised the question whether any bacterium that gains access to the cytosol of mammalian cells is able to replicate in this seemingly nutrient-rich compartment. To address this important issue, we developed a microinjection technique to directly place bacteria tagged with the green fluorescent protein (GFP) into the cytosol of epithelial cells and to study their capability of cytosolic multiplication.

## Materials and Methods

**Bacterial Strains and Plasmids.** The *L. monocytogenes* wild-type strain EGD and the isogenic mutants of this strain used in this study were previously described (8–14).The *Shigella flexneri iscA* mutant was a gift of Dr. Sansonnetti (Paris), and the EIEC strain W7062 was provided by U. Karch (Würzburg, Germany). All other bacterial strains are from our own culture collection.

For amplification of the *hpt* gene, we used the following primer pair: 5′-CGCAAGATAATGCTGCAGATAAGCGATTATATG-3′ and 5′-GCCATGCCGCTGCAGCTATTATGGGTGTCCTTT-3′. These primers were derived from the known *hpt* sequence of *L. monocytogenes* (I.C.-C., unpublished data). For cloning purposes, we introduced *Pst*I restriction sites into the primers. The amplified fragment contains the *hpt* promoter and the entire structural gene for *hpt.* The amplification of the DNA sequence by PCR was performed in a 100-$\mu$l reaction volume following standard protocols. This fragment was inserted into the *Pst*I site of pLSV16-$P_{actA}$-*gfp* (8), which resulted in the recombinant plasmid pJOE-$P_{actA}$-*gfp* carrying *hpt* with its own promoter and *gfp* under the control of the *actA* promoter, $P_{actA}$.

Strain EGD and its mutant derivatives were transformed by electroporation with the plasmid pLSV116-$P_{actA}$-*gfp* (8, 15), pKSBC16-$P_{sod}$-*gfp* (J. Daniels and A.B., unpublished data), or pJOE-$P_{actA}$-*gfp* as described (8, 15).

**Microinjection Procedure.** A total of 5–8 pl of a suspension of GFP-labeled bacteria in PBS buffer at a cell density of $10^6$ bacteria per ml was microinjected into a single Caco-2 cell. Bacteria were grown in BHI medium to a logarithmic state ($5 \times 10^8$ cells per ml), and the Caco-2 cells were cultured in Petri dishes (60 mm diameter) to semiconfluency in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine (GIBCO/BRL) at 37°C in a humidified 5% $CO_2$ atmosphere. Microinjection was performed with an automated transjector 5256 (Eppendorf) applying a pressure of 110 hPa. Borosilicate capillaries (Sutter Instruments, Novato, CA) were made with a capillary pipette puller (Bachofer, Reutlingen, Germany). The average inner diameter of the used pipettes was 0.7 $\mu$m. The quality of each pipette used was controlled by microscopy. The microinjection was carried out in the presence of 15 $\mu$g/ml gentamicin in the culture medium. After the microinjection of about 200 Caco-2 cells per a given assay, the

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Abbreviations: EIEC, enteroinvasive *E. coli*; LLO, lysteriolysin; GFP, green fluorescent protein; G-1-P, glucose-1-phosphate.

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culture medium was removed, cells were washed three times with PBS buffer, and fresh culture medium with 50 $\mu$g/ml gentamicin was added. At defined time points, the microinjected Caco-2 cells were analyzed by epifluorescence microscopy by using a Leica DM IRB microscope (Leica, Deerfield, IL). Double and triple images using emission filter wavelengths of 320, 460, and 525 nm and photographs were taken by using a MicroMAX CCD camera (Princeton Instruments, Trenton, NJ) and assembled using the METAMORPH imaging software (Universal Imaging, Media, PA).

**Test for Apoptosis or Necrosis of Microinjected Cells.** Caco-2 cells microinjected with the indicated bacteria or left uninfected were stained after 12 or 24 h with Hoechst 33342 (final concentration 15 $\mu$g/ml) for 20 min and with propidium iodide (final concentration 2 $\mu$g/ml) for 2–3 min and immediately photographed.

## Results

**The Procedure of Microinjecting Bacteria into the Cytosol of Caco-2 Cells.** To establish and standardize microinjection of bacteria into the cytosol of mammalian cells, we used the epithelial cell line Caco-2 as host for the facultative intracellular bacterium *L. monocytogenes.* Caco-2 cells were grown to a semiconfluent state characterized by the formation of large separated cell islands. In this growth state, the Caco-2 cells are tightly adherent to Petri dishes, which is essential for the successful microinjection of bacteria. The test bacterium, *L. monocytogenes* strain EGD, replicates after infection in the cytosol of Caco-2 cells (1, 2). To distinguish between bacteria microinjected into the host cell cytosol and bacteria released into the culture medium during the microinjection, we took advantage of our recent observation (8) that the expression of the *gfp* cDNA (encoding the GFP) under the control of the promoter for the listerial virulence gene *actA* is strongly activated in the host cell cytosol. This PrfA-dependent promoter ($P_{actA}$) shows, however, very low activity when the bacteria are grown in rich culture media or reside in the phagosome of infected host cells (8, 15). Hence, GFP-mediated fluorescence is expressed by listeriae that are injected into the host cell cytosol but not by those listeriae that are released during microinjection into the culture medium. In addition, we constructed a shuttle plasmid capable of replication in Gram-positive and Gram-negative bacteria, which carries the *gfp* cDNA behind the listerial superoxide dismutase (*sod*) promoter. This PrfA-independent promoter leads to constitutive, high levels of GFP expression in *L. monocytogenes* both within infected host cells and in cell culture media. The stability of both plasmids in the analyzed bacteria is high; when tested *in vitro*, the plasmid loss never exceeds 1% per ten generations. Expression of GFP does not alter cell viability or the doubling time of the used *L. monocytogenes* strains *in vitro*.

Microinjection of the bacteria into Caco-2 cells was performed by using an automated device that injects a constant volume of bacterial suspension into the host cell cytosol with the aid of specially manufactured needles. The distance between two microinjected Caco-2 cells was chosen to be at least ten cell diameters in the monolayer to allow observation of single cell events. To avoid possible infection of the Caco-2 cells by excess bacteria released into the culture medium rather than injected into host cells, the experiments were carried out in the presence of gentamicin to kill extracellular *L. monocytogenes*. As an additional control, the same number of bacteria microinjected into Caco-2 cells was added to an equal volume of gentamicin-containing culture medium with a similar number of Caco-2 cells. Infected Caco-2 cells were never observed in these control assays, probably because of the low multiplicity of infection (<0.005 bacteria per Caco-2 cell) and the presence of gentamicin in the culture medium. The efficiency of microinjection was close to 10%, i.e., about 20 of 200 cells that were routinely microinjected in each experiment (four independent microinjections for each strain) contained intracellular (fluorescent) bacteria. A single successfully microinjected Caco-2 cell carried an average of one to two bacteria directly after injection (zero time). About 50% of these bacteria performed subsequently efficient replication (more than 32 bacteria in the primary infected cell after 8 h postinjection) and cell spreading, whereas the others showed no or inefficient replication (less than 8 bacteria per cell) and no cell spreading. These latter inefficient replication events might be due to a lack of full recovery of the host cells and/or the bacteria after microinjection and a possible flow of antibiotic into the microinjected cells. For the calculation of replication efficiencies (see Table 1) the 50% successfully microinjected cells were always used, which showed the highest number of intracellular bacteria.

**Table 1. Efficiency of intracellular replication and cell-to-cell spread of *L. monocytogenes* wild-type strain EGD and isogenic mutants with in-frame deletions in various virulence genes**

| Strain (mutation in gene) | Replication efficiency* | Spreading efficiency† |
|---|---|---|
| None (wt) | 1.0 | 1.0 |
| *hly* | 0.6 (± 0.15)‡ | 0.01 (± 0.01)‡ |
| *actA* | 0.9 (± 0.2) | 0.0‡ |
| *plcA* | 0.9 (± 0.1) | 0.7 (± 0.2) |
| *plcB* | 0.7 (± 0.2) | 0.5 (± 0.2)‡ |
| *plcA* + *plcB* | 0.4 (± 0.1)‡ | 0.3 (± 0.15)‡ |
| *plcA* + *hly* + *mpl* + *actA* + *plcB* | 0.5 (± 0.1)‡ | 0.0‡ |
| *inlA* + *inlB* | 1.0 (± 0.05) | 1.0 (± 0.1) |
| *inlC* | 0.8 (± 0.1) | 0.7 (± 0.2) |
| *prfA* | 0.05 (± 0.01)‡ | 0.0‡ |
| *hpt* | 0.1 (± 0.02)‡ | 0.2 (± 0.1)‡ |

*Replication efficiency was determined 8 h postinjection of the corresponding strain. At this time, cell spreading into neighboring host cells was still at a low level, and the fluorescent bacteria within the primarily injected host cell could be easily seen and counted. The given values represent an average from 10 successfully microinjected Caco-2 cells in which efficient bacterial replication is seen (about 50% of all successfully microinjected cells). In such host cells microinjected with the wild-type strain (wt), >32 bacteria per microinjected cell was observed (giving a total of at least 320 bacteria per 10 cells). This value (32 or more bacteria per host cell within 8 h) was defined as replication efficiency 1.0, and the efficiency of replication of the mutant strains (all bacteria in 10 successfully injected cells, e.g., in *prfA* mutant a total of 16 bacteria) was calculated in relation to this value based on the counted intracellular bacteria (each experiment was performed four times; the numbers in parentheses mark the maximal deviation from the average value).

†Spreading efficiency was determined 24 h postinjection by counting all fluorescent bacteria within a focus surrounding the primary injected host cell. The given spreading efficiencies represent again average values based on bacterial counts from 20 microinjected Caco-2 cells with the surrounding spreading foci (if existing) related to the number of bacteria within the spreading foci of the wild-type strain (~500 bacteria per focus), which was again set to 1.0.

‡Statistically significant differences relative to the wild-type values, with $P <$ 0.01 as determined by Student's *t* test.

The microinjected *L. monocytogenes* wild-type strain EGD harboring a multicopy plasmid with the *gfp* cDNA under the control of the $P_{actA}$ is not or is only weakly fluorescent immediately after injection. It showed strong fluorescence 2 h postinjection, and bacterial replication started only 2–3 h postinjection (Fig. 1). None of the bacteria that were accidentally released into the culture medium during injection exhibited significant fluorescence. The successfully injected bacteria multiplied extensively in the cytosol of the Caco-2 cells within the next hours with doubling time of about 40 min and spread to neighboring cells (Fig. 1). Epithelial cells successfully microinjected with the wild-type strain remained morphologically intact and viable as shown by differential staining of the cells with Hoechst 33342 and propidium iodide (data not shown). An LLO-negative mutant (carrying an in-frame deletion in the *hly* gene) multiplied

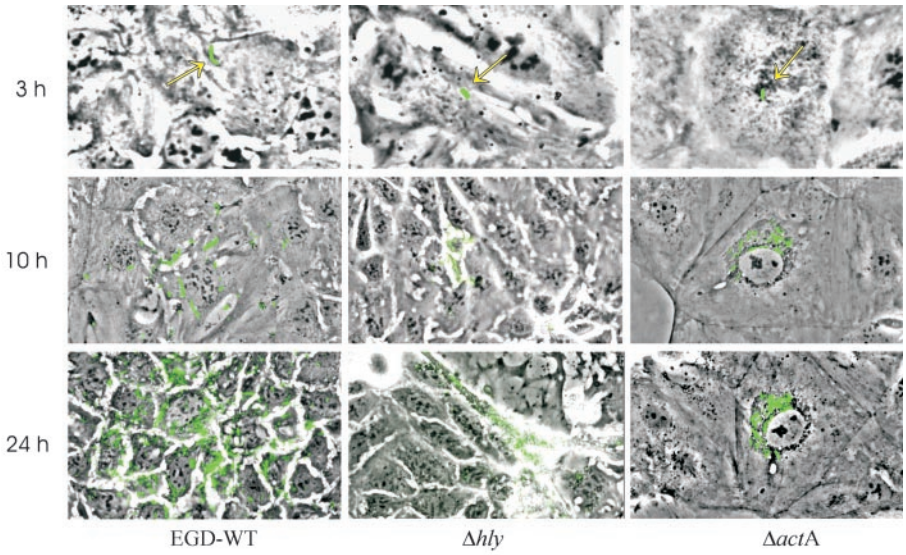


**Fig. 1.** Microinjection of wild-type (WT) and mutant strains (Δ*hly* and Δ*actA*) of *L. monocytogenes* into Caco-2 cells. Microinjection into Caco-2 cells was performed as described in *Materials and Methods*, using *L. monocytogenes* wild-type strain EGD and isogenic mutants with deletions in *hly* and *actA*. All three strains harbor the same plasmid carrying $P_{actA}$-*gfp*. Bacterial replication was determined after 3, 10, and 24 h. Arrows mark single fluorescent bacteria, which appear 3 h after microinjection.

in the cytosol of the injected Caco-2 cells (Fig. 1), indicating that there is no membrane surrounding the injected bacteria (Fig. 1, Table 1). Only a few single bacteria were observed in neighboring Caco-2 cells (see fluorescent bacteria indicated by the arrows in Fig. 1), suggesting that the *hly* mutant may still form spreading vacuoles but is unable to disrupt the vacuole formed during cell-to-cell spread. Likewise, the *act*A mutant [which is also impaired in cell-to-cell spread because of the inability of intracellular and intercellular movement (1, 2, 9, 10)] replicated in the cytosol of the injected cells at a similar rate as the wild-type strain (Fig. 1, Table 1). As expected, it did not spread into neighboring cells (Fig. 1). These data show that *L. monocytogenes* reproduces its normal behavior upon microinjection into the host cell cytosol, even if the passage through the phagosomal compartment is circumvented.

***S. flexneri* and Enteroinvasive *E. coli*, but Not Extracellular and Facultative Intracellular Bacteria That Normally Reside in a Vacuole, Replicate in the Cytosol of Caco-2 Cells.** Similar to *L. monocytogenes*, *Shigella* spp. and the closely related EIEC strains are known to replicate in the cytosol of infected host cells (16). An *ics*A mutant of *S. flexneri*, which is unable to spread to neighboring host cells (17), and the EIEC strain W7062 were transformed with the $P_{sod}$-*gfp*-carrying plasmid. The *Shigella* mutant replicated even more efficiently than *L. monocytogenes* after microinjection into Caco-2 cells, i.e., up to 70% of the successfully microinjected host cells (about 10% of the total microinjected cells) showed more than 64 bacteria per cell, and the generation time of Shigellae within the Caco-2 cells was only 30 min compared with 40 min observed for Listeriae. As expected by the *ics*A mutation, no cell-to-cell-spread was observed (Fig. 2). The EIEC wild-type strain also replicated in the host cell cytosol (Fig. 2), but replication was much less efficient than that of *S. flexneri*—i.e., the number of replicating bacteria rarely exceeded 32, and only about 30% of the successfully microinjected host cells showed replicating bacteria. Unexpectedly, spreading of these bacteria into neighboring cells was rarely observed. To determine whether this was a strain-specific defect, we tested three additional EIEC wild-type strains from different strain collections and obtained similar results. These data suggest that EIEC strains are apparently less proficient in cytosolic replication and cell spreading (at least in Caco-2 cells) than *S. flexneri*.

*S. enterica* serovar *Thyphimurium* and *L. pneumophila* multiply in specialized phagosomal compartments of phagocytic and nonphagocytic mammalian host cells (3, 4). We have previously shown (11) that *Salmonella typhimurium* expressing LLO can disrupt the phagosomal membrane, it reaches the cytosol, but it does not proficiently replicate. All of the above bacterial species were transformed with the $P_{sod}$-*gfp* plasmid with the exception of *L. pneumophila*. For the latter microorganism, *gfp* cDNA was placed under the control of the promoter of the *mip* gene (12). All strains exhibited strong fluorescence already under extracellular conditions. Expression of GFP did not significantly affect cell viability and replication rate *in vitro*. When the *S. typhimurium* strain was microinjected into Caco-2 cells, the injected bacteria showed strong fluorescence immediately upon injection, and fluorescence did not diminish during the monitored 24 h (Fig. 2). Multiplication was observed in none of the Caco-2 cells, which carried a single fluorescent bacterium during the entire 24-h observation period. Similar results were obtained after microinjection of GFP-tagged *L. pneumophila* (data not shown). It is unlikely that this failure of intracellular replication is caused by gentamicin present during the microinjection procedure because these latter Gram-negative bacteria are even less sensitive to the antibiotic than *L. monocytogenes*, and microinjection performed in the absence of gentamicin also did not show intracellularly growing bacteria. Although we cannot completely rule out the possibility that these latter bacteria are more sensitive to the manipulation by the microinjection than *L. monocytogenes*, *S. flexneri*, and EIEC, our data rather suggest that these intracellular bacteria are unable to grow in the cytosol of Caco-2 cells.

The inability of cytosolic replication of the latter bacteria after microinjection into Caco-2 cells was surprising in the light of previous work (7), which showed that even truly extracellular bacteria, like *B. subtilis*, replicate in the cytosol of macrophages when provided with listeriolysin to open the phagosomal compartment. We therefore microinjected prototrophic extracellular pathogenic and nonpathogenic bacteria including *Yersinia enterocolitica*, the well characterized uropathogenic *E. coli* strain 536 (18), and *B. subtilis* DSM 401 into Caco-2 cells. These bacterial strains were also

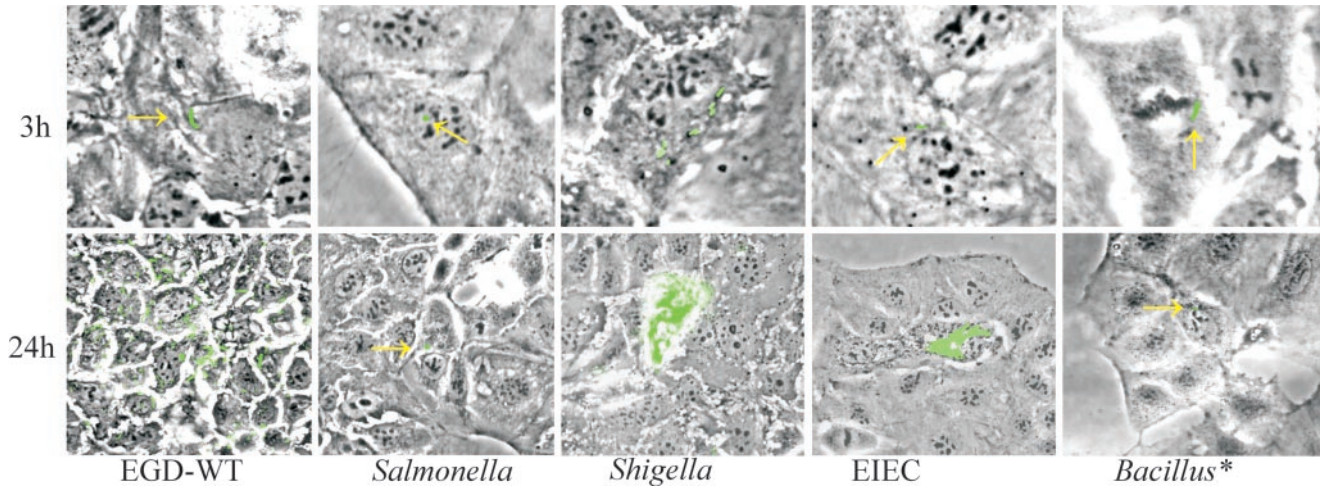


**Fig. 2.** Microinjection of Caco-2 cells with *L. monocytogenes* EGD, *S. typhimurium* 14028s, *S. flexneri iscA*, EIEC strain W7062, and *B. subtilis* DSM401. All strains were GFP-labeled by transformation with pKSBC16-$P_{sod}$-*gfp*. Bacteria grown overnight were used for microinjection. Intracellular bacterial replication was monitored until 24 h after injection. Photographs shown were taken at 3 and 24 h postinjection, except for *B. subtilis* (Bacillus*), which were taken at 1 and 6 h postinjection; in this case, there were hardly any fluorescent bacteria after 24 h.

labeled with the $P_{sod}$-*gfp*-carrying plasmid. GFP expression was observed in all of these strains. The results showed that none of these bacterial strains were able to replicate in the host cell cytosol after microinjection (data not shown). Single fluorescent *E. coli* and *B. subtilis* bacteria were usually observed up to 8 h postinjection, but most fluorescent bacteria disappeared after longer incubation times, suggesting that these bacteria are probably incapable of replication and *de novo* protein synthesis in the host cell cytosol. To test whether the lack of general cytosolic replication of these latter bacteria is specific for the epithelioid cell line Caco-2, we used also a hepatocytic cell line HepG2 and the macrophage cell line J774 for microinjection. Microinjection into these two cell lines yielded similar results as shown above for Caco-2 cells (data not shown).

We observed, however, that in a few host cells microinjected with *Y. enterocolitica*, *S. typhimurium*, and even *B. subtilis* and *L. innocua*, extensive bacterial replication takes place (Fig. 3) similar to what has been previously described (7). Interestingly, all of these cells (which represent $<1\%$ of the microinjected cells) had undergone cell death (probably apoptosis and/or necrosis, as shown by staining the cells with Hoechst 33342 and propidium iodide).

**Efficient Replication of *L. monocytogenes* in the Cytosol of Caco-2 Cells Requires a PrfA-Dependent Gene.** The results described above raise the following question: What are the factors that enable *L. monocytogenes*, *S. flexneri*, and enteroinvasive *E. coli*, but not the other bacteria, to replicate in the host cell cytosol? We started to address this important problem by examining the role of the virulence genes of *L. monocytogenes*, which have been identified in the past years (1, 2, 9, 10, 13, 19–23) for cytosolic replication. Most of these genes are part of a chromosomal gene cluster that is controlled by the transcriptional activator PrfA (19). To reach the cytosol of host cells, *L. monocytogenes* needs functional LLO and, to a lesser extent, the two phospholipases, PlcA and PlcB (5, 6). Because the expression of the genes encoding these products is strictly dependent on PrfA, a *prfA* mutant can also not reach the cytosol after infection of host cells; hence, the impact of these and/or possibly other (yet unknown) PrfA-regulated genes for replication of *L. monocytogenes* in the host cell cytosol cannot be studied by natural infection. The use of the microinjection technique circumvents this problem.

To test whether PrfA-dependent gene products are at all necessary for cytosolic replication, we first microinjected Caco-2 cells with the *prfA* mutant. This mutant strain, carrying an

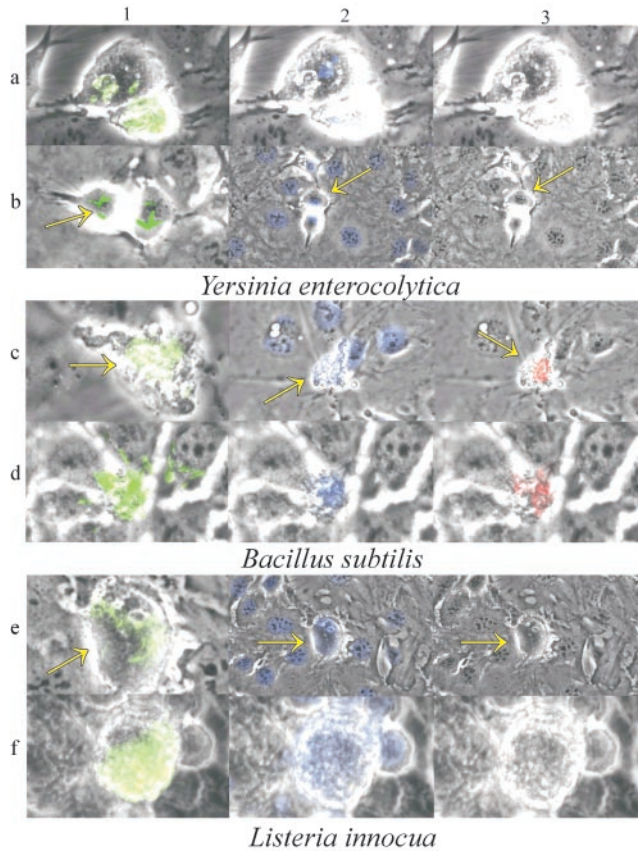


**Fig. 3.** Atypical replication of *Y. enterocolitica*, *B. subtilis*, and *L. innocua*, each carrying the $P_{sod}$-*gfp* plasmid in Caco-2 cells after microinjection. With a frequency of about 1 in 30 successfully microinjected cells, extensive replication of the bacteria were observed 12 to 24 h postinjection. These host cells were apoptotic or necrotic as demonstrated by staining with Hoechst 33342 (20 $\mu$g/ml) and propidium iodide (2 $\mu$g/ml) in contrast to those cells that carried a single bacterium. Note the lower magnification of panels 2b, 2c, 2e and 3b, 3c, 3e, which was used to visualize neighboring cells with intact nuclei for comparison. The Caco-2 cell shown in panel 1d has already lysed to some extent, and the fluorescent bacteria seen at the neighboring cells are not intracellular. This is occasionally seen when the host cells become necrotic. Arrows point to the same cells before and after staining with the Hoechst 33342 (panels 2a–2f) and propidium iodide (panels 3a–3f).

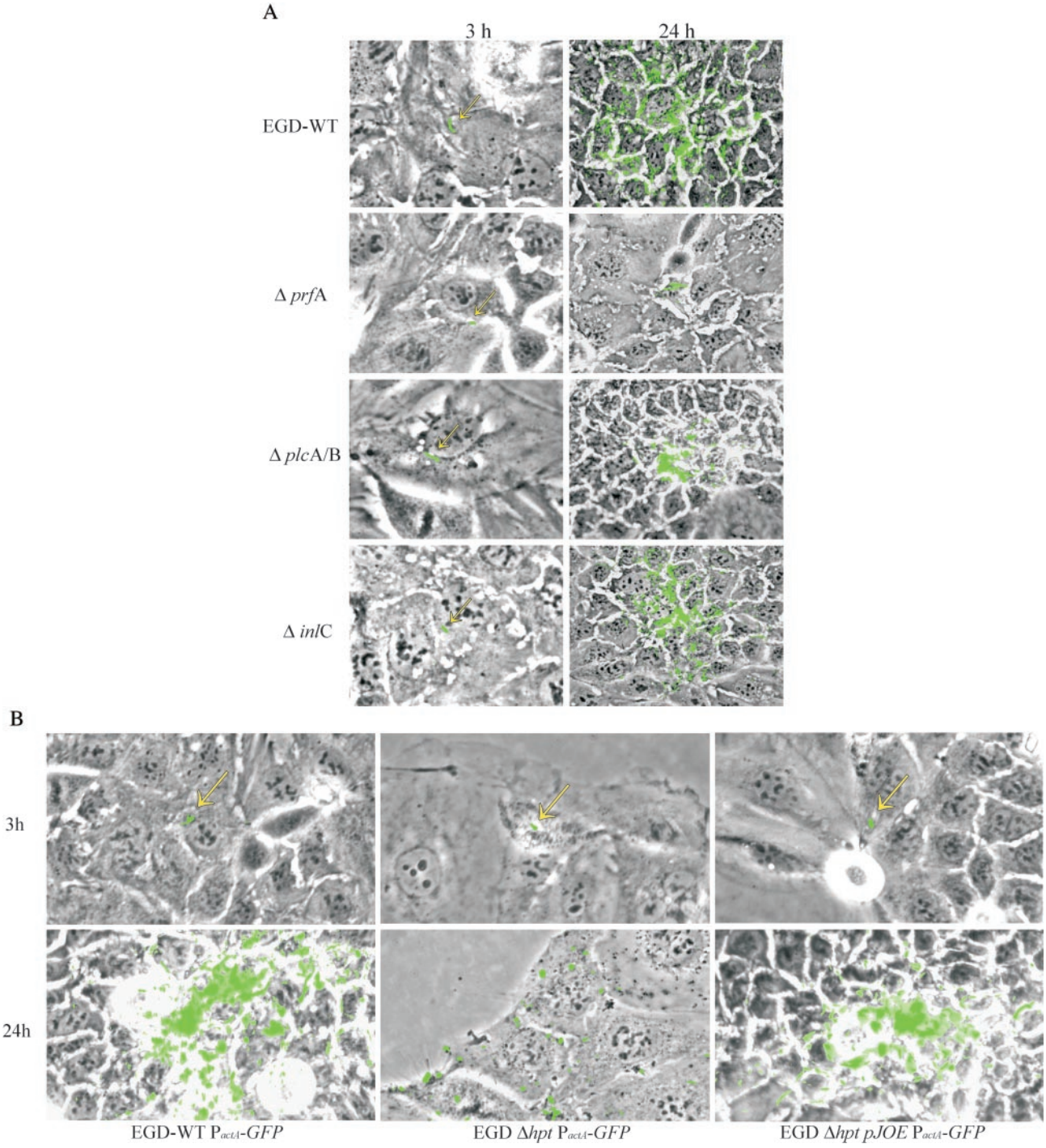


**Fig. 4.** (*A*) Microinjection of Caco-2 cells with *L. monocytogenes* EGD and the isogenic mutants with in-frame deletions in the PrfA-regulated genes, *prfA*, *plcA*, and *plcB*, and *inlC* and (*B*) with a mutant carrying an in-frame deletion in *hpt* gene and with this mutant complemented with a plasmid carrying the *hpt* gene under the control of its own PrfA-dependent promoter together with the $P_{actA}$-regulated *gfp* cassette (pJOE-$P_{actA}$-*gfp*). The isogenic EGD wild-type strain carrying a similar plasmid without the *hpt* gene was used as control. Intracellular bacterial replication was monitored for 24 h; photographs of microinjected Caco-2 cells shown were taken after 3 h and 24 h.

in-frame deletion in the *prfA* gene (14), was labeled with the $P_{sod}$-*gfp* plasmid. After microinjection, the *prfA* mutant strain showed a drastically reduced cytosolic replication (Fig. 4*A*). In many Caco-2 cells, the single injected bacterium did not replicate at all, whereas in some cells one or two bacterial cell divisions seemed to occur. These data suggest that PrfA-dependent gene product(s) of *L. monocytogenes* are essential for cytosolic replication. As already shown above, the *hly* and especially the *actA* mutant replicated with rates comparable to that of the wild-type strain when microinjected into the cytosol of Caco-2 cells. Similar results were obtained with mutants defective in the other known PrfA-dependent virulence genes, i.e., *plcA*, *plcB*, *inlC*, and *inlA/B* mutants (Fig. 4*A* and Table 1). Even a mutant in which the entire PrfA gene cluster except the *prfA* gene is deleted (14) showed a similar replication efficiency as the *plcA-plcB* mutant in Fig. 4*A* and Table 1. These findings ruled out the

known PrfA-dependent gene products for being responsible for the observed strong impairment of the *prfA* mutant in cytosolic replication and suggested that PrfA-dependent gene product(s) other than the known PrfA-dependent virulence factors were indispensable for cytosolic replication of *L. monocytogenes*.

**The PrfA-Regulated hpt Gene Encoding an Uptake System for Phosphorylated Sugars Is Required for Efficient Replication of *L. monocytogenes* in the Cytosol After Microinjection.** It has been reported that *L. monocytogenes* utilizes glucose-1-phosphate (G-1-P) in a strictly PrfA-dependent manner (24). G-1-P is a possible carbon source for intracellular growth of *L. monocytogenes* in mammalian host cells generated by degradation of glycogen (M.B., unpublished data). The gene *hpt*, responsible for G-1-P uptake in *L. monocytogenes* encoding a sugar phosphate permease, was recently identified. The gene has been shown to be tightly regulated by PrfA. An *L. monocytogenes* mutant with an in-frame deletion in *hpt* is unable to grow on G-1-P *in vitro* (I.C.-C., unpublished results). When microinjected in Caco-2 cells, this mutant strain showed strongly impaired replication in the cytosol; cell spreading occurred also at a reduced rate (Fig. 4*B* and Table 1). Cytosolic replication and cell spreading were restored to almost wild-type levels upon complementation with a plasmid carrying the *hpt* gene under the control of its PrfA-dependent promotor (Fig. 4*B*). These results suggest that G-1-P possibly may serve as a carbon source for growth of *L. monocytogenes* in the host cell cytosol, where uptake is facilitated by the PrfA-regulated *hpt* gene.

## Discussion

The microinjection technique described here allows the direct injection of single bacteria into the cytosol of mammalian cells, such as epithelial cells, hepatocytes, or macrophages. Our data obtained with this method show that the cytosol of a mammalian cell is not a rich nutrient broth that would support bacterial multiplication in general (7, 25). Indeed, only few bacteria are able to efficiently replicate in this compartment after microinjection. Interestingly, all bacteria found to be capable of cytosolic replication after microinjection are facultative intracellular pathogens, like *Shigella* spp., the related enteroinvasive *E. coli* strains, and *L. monocytogenes*, which are already naturally adapted to replication in the cytosol. These results lend support to the idea that the proper adaptation of the bacterial metabolism to the host cell environment is essential for successful replication in a specific host cell compartment (26). Obviously, only a few of the tested bacterial species fulfill the metabolic requirements for growth in the cytosol of mammalian cells. In *L. monocytogenes*, cytosolic replication seems to require the gene for a sugar phosphate transporter specifically controlled by the transcription factor, PrfA, which differentially regulates most of the known listerial virulence genes (1, 2, 15, 19). PrfA is known to be active particularly when the bacteria grow in the cytosol of host cells (8, 15), and the induced expression of this sugar uptake system by the host cell cytosol environment may enable *L. monocytogenes* to utilize glucose-1-phosphate (24), which is possibly derived from glycogen of the host cell. Similar uptake systems for phosphorylated sugars are also present in *E. coli* (27) and possibly other bacteria, but their expression in the cytosolic milieu of host cells may be less efficient than the PrfA-regulated uptake system of *L. monocytogenes.* Further experiments will show whether this PrfA-controlled uptake of glucose-1-phosphate is the only specific metabolic requirement for cytosolic growth of *L. monocytogenes*.

At a low frequency, Caco-2 cells harboring a large number of bacteria in the cytosol are observed with all tested bacterial species after microinjection (about 1% of the cells containing microinjected bacteria). All of these cells have undergone cell death. These rare events are observed at a rate that corresponds roughly to the rate of spontaneous apoptosis of Caco-2 cells under the culture conditions. We therefore assume that this atypical cytosolic replication occurs in apoptotic cells only and that such cells may provide more readily available resources for bacterial growth. The replicating bacteria in these cells are always seen around the cell nucleus, suggesting that components released from the apoptotic nucleus may serve as nutrients for bacterial growth. We suggest that the extensive cytosolic replication observed previously with LLO-expressing *B. subtilis* (7) and apathogenic *L. innocua* (28) might be also the result of such events. An alternative explanation could be that bacterial growth in the cytosol of intact host cells is restricted by antibacterial components, and this restriction is abolished in apoptotic (necrotic) cells (29).

The described technique of microinjecting bacteria directly into the cytosol of mammalian cells may help to further unravel in detail the specific requirements for bacterial replication in the host cell cytosol.

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