Bacillus subtilis expressing a haemolysin gene from Listeria monocytogenes can grow in mammalian cells

Jacek Bielecki†, Philip Youngman, Patricia Connelly* & Daniel A. Portnoy‡

Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6076, USA
*Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6018, USA
†Permanent address: Institute of Microbiology, Warsaw University, Nowy Świat 67, 00-046 Warsaw, Poland.
‡To whom correspondence should be addressed.

INTRACELLULAR parasites can be classified into those that reside within a host vacuole and those which grow directly in the host cytoplasm. Members of the latter group, which includes Rickettsia†, Shigella§, Treponema cruzi§ and Listeria monocytogenes‡, possess haemolytic activity associated with the ability to enter the host cytoplasm. Therefore mutants of L. monocytogenes lacking a pore-forming haemolysin, listeriolysin O, do not escape from the endosomal compartment and consequently fail to become established in the cytoplasm. To examine the role of listeriolysin O, we cloned the structural gene for the L. monocytogenes haemolysin, hlyA, into an asparaginase mutant of Bacillus subtilis under the control of an IPTG-inducible promoter. To be internalized by the macrophage-like cell line J774, haemolytic B. subtilis disrupted the phagosomal membrane and grew rapidly within the macrophage cytoplasm. These results show that a single gene product is sufficient to convert a common soil bacterium into a parasite that can grow in the cytoplasm of a mammalian cell.

The structural gene for the L. monocytogenes haemolysin, hlyA, was cloned on a 2.5-kilobase (kb) Sau96I fragment into the HindIII site of plasmid pAG58-ble-1 downstream from the IPTG-inducible SPAC cassette. This expression cassette was then integrated into the chromosome of a nonreverting sporation-deficient mutant of B. subtilis by homologous recombination. The resulting strain, B. subtilis (hlyA), secreted haemolytic activity only during growth in the presence of IPTG (data not shown). After induction, this strain secreted about the same haemolytic activity (80 units ml⁻¹) as wild-type L. monocytogenes grown in the same conditions. A new polypeptide was also secreted only after IPTG-induction and co-migrated with authentic listeriolysin O (of relative molecular mass (M_r) of 38,000) as seen by SDS-PAGE (data not shown). These data indicate that a single gene product is sufficient for production and secretion of haemolytic activity by B. subtilis, and that this activity depends on induction of the SPAC promoter.

To investigate the role of haemolysin in establishment of intracellular growth we challenged the phagocytic macrophage-like cell line J774 with B. subtilis (hlyA). Extracellular multiplication was prevented by the addition of gentamicin after 1 h. To our surprise, B. subtilis (hlyA), when grown and used to infect the cells in the presence of IPTG, multiplied inside the macrophages with an intracellular doubling time of about 1 h. This is the same intracellular doubling time as L. monocytogenes (ref. 8 and Fig. 2). In the absence of IPTG, B. subtilis was internalized and killed to some extent, and no growth was detectable (Fig. 2). In addition, IPTG could be added after internalization (t = 2) and the surviving intracellular bacteria commenced growth (data not shown). This suggests that bacteria residing in a vacuole were capable of de novo protein synthesis.

FIG. 1 Physical map of sequences integrated into the B. subtilis chromosome that place expression of the L. monocytogenes hlyA gene under control of the IPTG-inducible P_ape promoter. Cross-hatched box (lacO), repressor-binding sequences from the Escherichia coli lac operator region; P_ape, strong promoter derived from B. subtilis phage SP01 whose expression is constitutive except when lac repressor is bound to lacO; hlyA, DNA fragment containing the entire coding sequence of listeriolysin O but lacking a promoter that can function in B. subtilis; ble, gene conferring resistance to bleomycin; cat, gene conferring chloramphenicol resistance in B. subtilis.

FIG. 2 Growth of hly− and hly+ L. monocytogenes and B. subtilis in the J774 macrophage-like cell line. Hly− L. monocytogenes ( ), hly+ L. monocytogenes ( ), B. subtilis (hlyA) with IPTG (○); and B. subtilis (hlyA) without IPTG ( ).

METHODS. Growth of L. monocytogenes in J774 cells has been described previously. B. subtilis was grown at 37 °C to an optical density of 0.5 at 600 nm with or without 10 μM IPTG. J774 cells grown on coverslips in a 60-mm petri dish in a volume of 5 ml tissue culture medium were infected with 5 μl B. subtilis culture with or without 10 μM IPTG. After 30 min, the monolayers were washed three times with 37 °C PBS (pH 7.4) and fresh medium was added without IPTG. All other procedures have been described elsewhere. Error bars represent standard deviation of bacteria per coverslip in triplicate.

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even though they were unable to multiply. Cells were also challenged under the same conditions with *B. subtilis* harbouring the vector alone, and no bacterial growth was observed (data not shown).

Light microscopy of stained preparations revealed extensive intracellular multiplication by the haemolytic *B. subtilis* strain (Fig. 3). But unlike *L. monocytogenes*, which spreads to the periphery of infected cells and then from cell to cell13, haemolytic *B. subtilis* showed no evidence of such cell to cell spread and grew until the infected host cell eventually lysed. This was reflected in the decrease in colony-forming units seen at later times (Fig. 2). Electron microscopy revealed that some of the haemolytic *B. subtilis* were clearly growing freely in the host cytoplasm, whereas others appeared intact in vacuoles (Fig. 4a). By contrast, all of the *hly* *B. subtilis* were in vacuoles (Fig. 4b). These data indicate that haemolysin alone mediates lysis of the host vacuole, although it cannot be ruled out that another *B. subtilis* gene product acts with the *L. monocytogenes* haemolysin.

Haemolytic *L. monocytogenes* normally grows in the macrophage cytoplasm surrounded either by a cloud or a long tail of actin filaments, and this is thought to be a necessary prerequisite for cell-to-cell spread6. But it is still not known whether bacteria or host mediates actin filament polymerization. It was therefore of interest to determine whether *B. subtilis* growing in the host cytoplasm becomes associated with host actin filaments. Electron micrographs show that intracytoplasmic *B. subtilis* grow without actin filaments (Fig. 4a). These results indicate that the expression of haemolysin is sufficient to mediate access to the host cytoplasm, but other, so far undefined, *L. monocytogenes* gene products are required to nucleate actin polymerization.

Although it was not unexpected that haemolytic *B. subtilis* could lyse the phagosomal membrane, it was surprising that the bacteria grew within the cytoplasm. Thus, a single gene product is sufficient to convert a common soil organism into an intracellular parasite *in vitro*. This implies that the mammalian cytoplasm is a very permissive environment for bacterial growth. In contrast to the situation *in vitro*, haemolytic *B. subtilis* was absolutely avirulent after intravenous injection in BALB/c mice (D. Hinrichs, personal communication). This is consistent with the multifactorial nature of bacterial pathogenicity14. Clearly, one gene does not convert an organism into a pathogen. Nevertheless, the results of this study indicate that the evolutionary leap from an extracellular existence to an intracellular lifestyle may only require the acquisition of a limited number of genes.

Haemolytic activity has been observed in a wide variety of viral, bacterial and protozoan pathogens. The haemolysin now under study, listeriolysin O, is a member of a family of thiol-activated cytolysins present in 15 diverse species of Gram-positive bacteria which in addition to *Listeria* include members of the genera *Bacillus, Clostridium* and *Streptococcus*15. Many of these bacteria are pathogenic including *S. pyogenes, S. pneumoniae, C. perfringens* and *C. tetani*. The study presented here raises the intriguing possibility that other bacteria may at some stage in their life-cycle adopt an intracytoplasmic niche.