Identification and characterization

of the *inlGHE* gene cluster

of *Listeria monocytogenes*

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Diana Raffelsbauer

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Mitglieder der Promotionskommission:

Vorsitzender: *Prof. Dr. R. Kaldenhoff*

1. Gutachter: *Prof. Dr. W. Goebel*

2. Gutachter: *Prof. Dr. G. Krohne*

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Abgesehen von "Diplom-Biologin Univ." habe ich keine weiteren akademischen Grade erworben oder versucht zu erwerben.

Würzburg, Juli 2001

Diana Raffelsbauer

For my grandmother Estela, who was for me the greatest example of benevolence, strong will and determination.

A minha avó Estela, que foi para mim o maior exemplo de benevolência, força de vontade e determinação.

Acknowledgments

Abstract

Listeria monocytogenes is a facultative intracellular bacterium which is able to induce its own uptake into normally non-phagocytic mammalian cells. This internalization process is mediated by the internalins InlA and InlB, which belong to a growing family of leucine-rich repeat (LRR) proteins.

In the present study, a new gene cluster of *L. monocytogenes* EGD containing three internalin genes was identified and characterized. These genes, termed *inlG*, *inlH* and *inlE*, encode proteins of 490, 548 and 499 amino acids, respectively, which belong to the class of large, surface-bound internalins. Sequence of the proteins InlG, InlH and InlE revealed the presence of the characteristic features of the internalin protein family: a signal sequence, two regions of repeats (LRRs and B repeats), an inter-repeat region, and a putative cell wall anchor sequence containing the sorting motif LPXTG. InlG contains four LRR units of 22 amino acids, two B repeats of 70 and 49 amino acids, and the cell wall anchor sequence LPKTS at the C-terminus. InlH and InlE possess both seven LRRs and the anchor sequences LPTAG and LPITG, respectively. Whereas InlH has also two B repeats of 70 and 49 amino acids, InlE has only one B repeat of 47 amino acids, and in addition a third domain, termed region D, which is composed of two repeats of 20 amino acids each with high similarities to the B repeats.

The *inlGHE* gene cluster is flanked by two house-keeping genes encoding proteins homologous to the 6-phospho-β-glucosidase and the succinyl-diaminopimelate desuccinylase of *E. coli*. A similar internalin gene cluster, *inlC2DE*, localized to the same position on the chromosome was recently described in a different isolate (Dramsi *et al*., 1997). Sequence comparison of the two gene clusters indicates that *inlG* is an additional new internalin gene, whereas *inlH* is generated by a site specific in-frame deletion removing the 3'-terminal end of *inlC2* and the 5'-terminal part of *inlD*. The sites of recombination are located in the almost identical first B repeats of *inlC2* and *inlD*. The third gene of the *inlGHE* cluster, *inlE*, is nearly identical to the previously reported *inlE* gene (Dramsi *et al*., 1997). PCR analysis revealed the presence of the new genes *inlG* and *inlH* in most *L. monocytogenes* serotypes. In contrast, the *inlC2DE* could not be detected in any of the strains tested, suggesting that this cluster is specific for the strain used in the previous study. The occurrence of the *inlGHE* gene cluster was also examined in six *L. monocytogenes* clinical isolates, which were recently isolated from food or patients suffering from septicaemia or gastroenteritis. Four from these isolates show to possess the *inlGHE* cluster, but variations within the sequences of these genes were evident.

The promoter activity of each of the genes *inlG*, *inlH* and *inlE* was determined by measuring the fluorescence mediated by GFP-based expression plasmids. The *inlG* gene has the most active promoter, but lower activities were also detected with the *inlH* and *inlE* promoters. Transcription of the three *inl* genes seems to be independent of the transcriptional regulator PrfA and not induced after a shift from the rich growth medium BHI into the minimum essential medium MEM. In addition, the genes *inlG*, *inlH* and *inlE* are not expressed intracellularly in Caco-2 cells nor after contact of the bacteria to HepG-2 cells.

To investigate the role of the *inlGHE* gene cluster in virulence of *L. monocytogenes*, an *inlGHE* in-frame deletion mutant was constructed. Deletion was achieved by double

cross-over using a pLSV1-based knock-out plasmid which led to the fusion of the 5' end of *inlG* to the 3' end of *inlE*, resulting in the excision of the intervening sequence from the chromosome. When tested in the mouse model, the Δ*inlGHE* mutant exhibited after oral infection a significant reduction in bacterial counts in liver and spleen in comparison to the wild type strain, which argues for an important role of the *inlGHE* gene cluster in virulence.

In vitro studies using GFP-based expression plasmids revealed that the intracellular growth and cell-to-cell spreading of the Δ*inlGHE* strain in Caco-2 monolayers were not impaired. However, invasiveness of this mutant into non-phagocytic cells of the cell lines Caco-2 and TIB73 was increased by a factor of 2 to 3, whereas the adherence and invasion rates into professional phagocytes of the cell lines J774 and P388 were similar to that of the wild type strain. To examine whether deletion of the single genes from the *inlGHE* gene cluster has the same stimulatory effect on invasiveness as deletion of the complete gene cluster, the single deletion mutants Δ*inlG*, Δ*inlH* and Δ*inlE* were constructed by double cross-over. These mutants were subsequently reverted to the wild type by introducing a copy of the corresponding intact gene into the chromosome using knock-in plasmids.

To determine a putative contribution of the proteins InlG, InlH and InlE in combination with other internalins to the entry of *L. monocytogenes* into mammalian cells, the *inl* deletion mutants Δ*inlA/GHE*, Δ*inlB/GHE*, Δ*inlC/GHE*, Δ*inlA/B/GHE*, Δ*inlB/C/GHE*, Δ*inlA/C* and Δ*inlA/C/GHE* were constructed in the present study. Cellular invasion assays performed within the frame of a doctoral thesis by B. Bergmann using these mutants revealed that InlA- but not InlB-mediated internalization of *L. monocytogenes* into non-phagocytic cells additionally requires the internalins InlB, InlC, InlG, InlH and InlE (Bergmann, Raffelsbauer *et al.*, manuscript submmited).

Using the semi-quantitative RT-PCR technique, transcription of the three genes *inlG*, *inlH* and *inlE* was detected. Reactions using primers derived from *inlG* yielded higher amounts of product, suggesting that this gene is transcribed at higher efficiency than the other two genes. Deletion of single genes from the *inlGHE* cluster did not seem to affect transcription of the remaining genes, as RT-PCRs performed with the single deletion mutants yielded the same amount of product than the wild type strain.

Transcription of the *inlA*, *inlB* and *inlC* genes in the logarithmic growth phase was examined in different *inl* deletion mutants by RT-PCR. Deletion of *inlGHE* enhances transcription of the genes *inlA* and *inlB* but not of *inlC*. This enhancement is not transient, but can be detected at different time points of the bacterial growth curve. In addition, deletion of *inlA* increases transcription of *inlB* and vice-versa. An induction of transcription of these genes could be observed in the internalin mutant strains lacking *inlGHE*, *inlA* and/or *inlB*. In contrast, the amounts of *inlA* and *inlB* transcripts in the single mutants Δ*inlG*, Δ*inlH* and Δ*inlE* were similar to those from the wild type strain.

Infection of Caco-2 and J774 cells with *L. monocytogenes* EGD does not seem to elicit cell death by apoptosis. In J774 cells no significant alteration in the amount of histone-associated DNA fragments was observed between strains EGD and Δ*inlGHE*, which was only slightly increased in comparison to the non-infected control cells. However, infection of Caco-2 cells with two *L. monocytogenes* clinical isolates leads to a two- to three-fold increase in the level of DNA fragments in comparison to strain EGD. A correlation between the ability to induce apoptosis and the bacterial invasiveness was observed for the strains tested, including the Δ*inlGHE* mutant.

Zusammenfassung

Listeria monocytogenes ist ein fakultativ intrazelluläres Bakterium, das seine Aufnahme in normalerweise nicht-phagocytische Säugerzellen selbst induzieren kann. Dieser Internalisierungsprozess wird durch die Internaline InlA und InlB vermittelt, die zu einer wachsenden Familie von Leucin-reichen Repeat Proteinen gehören.

In der vorliegenden Arbeit wurde ein neues Gencluster mit drei Internalingenen identifiziert und charakterisiert. Diese als *inlG*, *inlH* und *inlE* bezeichneten Gene codieren für Proteine mit 490, 548 bzw. 499 Aminosäuren, die zur Klasse der großen, Oberflächengebundenen Internalinen gehören. Die Sequenz der Proteine InlG, InlH und InlE zeigt die charakteristischen Merkmale der Proteinfamilie der Internaline: eine Signalsequenz, zwei Repeat-Regionen (LRRs und B-Repeats), eine Inter-Repeat-Region sowie eine mögliche Zellwandankersequenz mit dem Motiv LPXTG. InlG enthält vier LRRs von je 22 Aminosäuren, zwei B-Repeats von 70 und 49 Aminosäuren und die Zellwandankersequenz LPKTS am C-Terminus. InlH und InlE besitzen beide sieben LRRs und die Ankersequenzen LPTAG bzw. LPITG. Während InlH auch zwei B-Repeats von 70 und 49 Aminosäuren enthält, hat InlE lediglich ein B-Repeat von 47 Aminosäuren. Zusätzlich besitzt InlE eine dritte, als Region D bezeichnete Domäne, die aus zwei Repeats von je 20 Aminosäuren mit hoher Ähnlichkeit zu den B-Repeats besteht.

Das *inlGHE*-Gencluster wird von zwei Haushaltsgenen flankiert, die für Proteine mit Homologie zu der 6-Phospho-β-Glucosidase bzw. Succinyl-Diaminopimelat-Desuccinylase von *E. coli* kodieren. Ein weiteres Internalin-Gencluster, *inlC2DE*, das an der gleichen Position auf dem Chromosom lokalisiert ist, wurde kürzlich in einem anderen *L. monocytogenes* Isolat identifiziert (Dramsi *et al.*, 1997). Sequenzvergleiche der beiden Genclusters zeigten, daß *inlG* ein zusätzliches, neues Internalin-Gen ist, während *inlH* durch eine in-frame Deletion des 3'-Endes von *inlC2* und des 5'-Teils von *inlD* entstanden ist. Die Rekombination erfolgte zwischen den nahezu gleichen ersten B-Repeats von *inlC2* und *inlD*. Das dritte Gen des Clusters, *inlE*, ist fast identisch mit dem vorherigen identifizierten *inlE*-Gen (Dramsi *et al.*, 1997). Durch PCR-Analyse wurden die neuen Gene *inlG* und *inlH* in den meisten *L. monocytogenes* Serotypen nachgewiesen. Im Gegensatz konnte das *inlC2DE*-Gencluster in keinem der getesteten Stämme detektiert werden. Das Vorkommen des *inlGHE*-Genclusters wurde auch in sechs *L. monocytogenes* klinischen Isolaten untersucht, die kürzlich aus Nahrungsmitteln oder Sepsis- und Gastroenteritis-Patienten isoliert wurden. Vier von diesen Isolaten besitzen das *inlGHE*-Gencluster, aber Sequenzvariationen innerhalb dieses Clusters wurden offensichtlich.

Die Promotoraktivität der Gene *inlG*, *inlH* und *inlE* wurde mit Hilfe von GFP-Expressionsplasmiden untersucht. Das *inlG*-Gen hat den aktivsten Promotor, aber geringere Aktivitäten wurden auch bei den *inlH*- und *inlE*-Promotoren gemessen. Die Transkription der drei Gene scheint PrfA-unabhängig und durch ein MEM-Shift nicht induzierbar zu sein. Die Expression der Gene *inlG*, *inlH* und *inlE* wird weder intrazellulär in Caco-2-Zellen noch durch Kontakt mit HepG-2-Zellen induziert.

Um die Rolle des *inlGHE*-Genclusters in der Virulenz von *L. monocytogenes* zu untersuchen, wurde eine *inlGHE* in-frame Deletionsmutante hergestellt. Die Deletion wurde durch double cross-over unter Verwendung eines pLSV1-basierten Knock-out-Plasmids herbeigeführt. Dabei wurde das 5'-Ende von *inlG* mit dem 3'-Ende von *inlE* in-frame fusioniert, und die dazwischen liegende Sequenz aus dem Chromosom ausgeschnitten. Im Mausmodell zeigte die Δ*inlGHE*-Mutante nach oraler Infektion eine signifikante Reduktion der Bakterienzahl in der Leber und Milz, die auf eine wichtige Rolle des *inlGHE*-

Genclusters in der Virulenz hindeutet.

In vitro-Studien mit GFP-Expressionsplasmiden zeigten, daß die intrazelluläre Replikation und Zell-zu-Zell-Spreading der $\Delta inlGHE$-Mutante nicht beeinträchtigt sind. Dennoch ist die Invasionsfähigkeit der Mutante in nicht-phagocytische Zellen der Zellinien Caco-2 und TIB73 um den Faktor zwei bis drei erhöht, während die Adhärenz und Invasion in professionelle Phagocyten der Zellinien J774 und P388 ähnlich sind wie die des Wildtyps. Um zu untersuchen, ob die Deletion von Einzelgenen des *inlGHE*-Genclusters einen ähnlichen stimulatorischen Effekt auf die Invasivität ausübt wie die Deletion des kompletten Genclusters, wurden die Einzelmutanten $\Delta inlG$, $\Delta inlH$ und $\Delta inlE$ durch double cross-over hergestellt. Diese Mutanten wurden anschließend zum Wildtyp revertiert, indem eine Kopie des entsprechenden intakten Gens mit Hilfe von Knock-in-Plasmiden eingeführt wurde.

Um einen möglichen Einfluß der Proteine InlG, InlH und InlE in Kombination mit anderen Internalinen auf die Aufnahme von *L. monocytogenes* in Säugerzellen zu untersuchen, wurden die Internalindeletionsmutanten $\Delta inlA/GHE$, $\Delta inlB/GHE$, $\Delta inlC/GHE$, $\Delta inlA/B/GHE$, $\Delta inlB/C/GHE$, $\Delta inlA/C$ und $\Delta inlA/C/GHE$ hergestellt. Zellinvasionsexperimente, die im Rahmen der Doktorarbeit von B. Bergmann mit Verwendung dieser Mutanten durchgeführt wurden, zeigten, daß die InlA- aber nicht InlB-vermittelte Internalisierung von *L. monocytogenes* in nicht-phagocytische Zellen zusätzlich die Internaline InlB, InlC, InlG, InlH und InlE benötigt (Bergmann, Raffelsbauer *et al*., manuscript submmited).

Mit Hilfe der semi-quantitativen RT-PCR-Technik wurde die Transkription der Gene *inlG*, *inlH* und *inlE* nachgewiesen. Reaktionen mit von *inlG* abgeleiteten Oligonukleotiden ergaben höhere Mengen an Produkten, was auf eine stärkere Transkription des *inlG*-Gens im Vergleich zu *inlH* und *inlE* hindeutet. Deletion der einzelnen Gene des *inlGHE*-Genclusters scheint die Transkription der restlichen Gene nicht zu beeinflussen, denn die mit Einzeldeletionsmutanten durchgeführten RT-PCRs ergaben eine vergleichbare Menge an Produkt wie die mit dem Wildtyp.

Die Transkription der Gene *inlA*, *inlB* und *inlC* aus verschiedenen Internalindeletionsmutanten in der logarithmischen Wachstumsphase wurde durch RT-PCR untersucht. Deletion von *inlGHE* erhöht die Transkription der Gene *inlA* und *inlB*, aber nicht *inlC*. Diese Erhöhung ist nicht vorübergehend, sondern kann zu verschiedenen Zeitpunkten der bakteriellen Wachstumskurve nachgewiesen werden. Zusätzlich führt die Deletion von *inlA* zu einer Erhöhung der Transkription von *inlB* und umgekehrt. Eine Induktion der Transkription dieser Gene konnte in den *inlGHE*-, *inlA*- und/oder *inlB*-Deletionsmutanten festgestellt werden. Im Gegensatz, die Mengen an *inlA*- und *inlB*-Transkripten in den Einzelmutanten $\Delta inlG$, $\Delta inlH$ und $\Delta inlE$ waren ähnlich wie die des Wildtyps.

Infektion von Caco-2- und J774-Zellen mit *L. monocytogenes* EGD induziert keinen Zelltod durch Apoptose. In J774-Zellen konnte kein signifikanter Unterschied in der Menge an Histon-assoziierten DNA-Fragmenten zwischen den Stämmen EGD und $\Delta inlGHE$ nachgewiesen werden, und im Vergleich zu nicht-infizierten Kontrollzellen war diese Menge nur leicht erhöht. Im Gegensatz dazu führt die Infektion von Caco-2-Zellen mit zwei *L. monocytogenes* klinischen Isolaten zu einer zwei- bis dreifachen Erhöhung in der Menge an DNA-Fragmenten im Vergleich zum Stamm EGD. Eine Korrelation zwischen der Fähigkeit, Apoptose zu induzieren, und der Invasivität der Bakterien konnte für die getesteten Stämme, einschließlich der $\Delta inlGHE$ Mutante, festgestellt werden.

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Introduction

Listeria monocytogenes is a Gram-positive, food-borne pathogenic bacterium responsible for serious localized and generalized infections such as meningitis, meningoencephalitis, abortion, stillbirth, septicaemia and occasionally gastroenteritis (Gray and Killinger, 1966; Lorber, 1996). Humans infected by *L. monocytogenes* are mainly immunocompromised people like AIDS and cancer patients, pregnant women, neonates and elderly people (reviewed by Schuchat *et al*., 1991). These infections lead to relatively high mortality rates, reaching 30% in adults and over 60% in neonates. The incidence of listeriosis seems to increase worldwide, with the number of cases rising especially in industrialized countries (Schuchat *et al*., 1991). This reflects the growth of the susceptible population due to the appearance of AIDS and the use of immunosuppressive medications in the treatment of malignancy and the management of organ transplantations, together with the increase in consume of types of foods in which *L. monocytogenes* is able to survive and grow.

Wide spread in the nature, *L. monocytogenes* is found in soil, water, plants, silage and also in human and animal feces (reviewed by Farber and Peterkin, 1991). It has been isolated from domesticated animals such as cattle, sheep, goat and poultry, but less frequently from wild animals. The natural habitat of the bacteria is decomposing material, in which they live as saprophytes. *L. monocytogenes* is able to grow in a wide range of temperatures between 1 and 45°C, reaching an optimum growth between 30 and 37°C (Schuchat *et al*., 1991). It is motile when grown in temperatures between 20 and 25°C, but non-motile at 37°C as result of the repression of flagellin synthesis.

L. monocytogenes is well equipped to survive in the environment and also to resist to usual food processing technologies, since it tolerates high concentrations of salt, relatively low pH values and low temperatures (Vazquez-Boland *et al*., 2001). The bacteria are even able to replicate at refrigeration temperatures, which makes *L. monocytogenes* a risk microorganism for the food industry. The foods most frequently implicated in cases of human listeriosis are dairy products including milk, soft cheeses, pâtés, sausages, meats, smoked fish, raw vegetables, salads and a variety of industrial ready-to-eat products. From beginning of the 1980s onwards a series of epidemic food-borne outbreaks of listeriosis in humans in North America and Europe has been reported which awoke the interest of governments and food manufacturers in this pathogenic bacterium. Based on somatic (O) and flagellar (H) antigens, *L. monocytogenes* is divided into 12 different serotypes, from which only three, namely serotypes 1/2a, 1/2b and 4b, account for more than 90% of human and animal cases of listeriosis (Schuchat *et al*., 1991). Whereas serovar 4b predominates in Europe, infections in the USA and Canada seem to be caused equally by serovars 1/2a, 1/2b and 4b.

L. monocytogenes belongs to a genus which currently includes five other species:

L. ivanovii, *L. innocua*, *L. seeligeri*, *L. welshimeri* and *L. grayi*. From these species only *L. monocytogenes* and *L. ivanovii* are potential pathogens (Vazquez-Boland *et al.*, 2001). Whereas *L. monocytogenes* is able to infect both humans and animals, infections caused by *L. ivanovii* are mostly limited to animals and human cases are rare. Listeriae are Gram-positive, rod-shaped, facultatively anaerobic, non-sporulating, catalase positive, oxidase negative bacteria of low G+C content closely related to *Bacillus*, *Streptococcus* and *Staphylococcus*.

For many years, *L. monocytogenes* received attention as model pathogen to study T cell-mediated immunity and most of the available knowledge of listeriosis comes from immunological studies performed in the past decades using the mouse model (Mackaness, 1962). An overview of the course of human listeriosis is shown in Fig. 1.1. Infection caused by *L. monocytogenes* begins with ingestion of contaminated food (Schlech *et al.*, 1983; Farber and Peterkin, 1991). After translocation of the gut epithelium, which seems to occur equally via enterocytes or M cells (Pron *et al.*, 1998), bacteria are taken up by the phagocytic cells underlying the Peyer's patches, where an early bacterial replication occurs (Racz *et al.*, 1972). Within these cells *L. monocytogenes* is transported via the lymph and the blood to the spleen and liver. In the latter organ, most of the bacteria are killed at an early stage of infection by resident macrophages (Kupffer cells), though a small fraction of the bacteria reaches the hepatocytes, where they induce apoptosis with concomitant release of chemoattractants which leads to an influx of neutrophils (Conlan and North, 1991; Rogers *et al.*, 1996). These phagocytic cells ingest bacteria and apoptotic hepatocytes and promote a rapid clearance of the infection. However, in the case of immunocompromised patients, bacteria replicate unrestrictedly after uptake into hepatocytes, from which they disseminate to brain and placenta (Rosen *et al.*, 1989). Infection of the central nervous system and the fetus by *L. monocytogenes* is due to the capacity of this bacterium to cross the blood-brain and the placental barriers. Infection of these two organs occurs either by induction of bacterial uptake directly from the blood into endothelial cells or by heterolog bacterial spreading from infected macrophages. Thus, a fundamental aspect of the pathogenicity of *L. monocytogenes* is its ability to invade and replicate in phagocytic and non-phagocytic cells.

In most infected tissues, *L. monocytogenes* has an intracellular location. Entry into non-phagocytic cells may allow traversal of tissue-specific barriers, such as the intestinal, blood-brain and placental barriers, and in addition promote survival of the pathogen by providing access to a nutrient-rich environment which is protected from host antibody or complement (Cossart and Lecuit, 1998). Intracellular pathogens have evolved different strategies to induce their own uptake into mammalian cells which are normally non-phagocytic. This entry process requires the participation of both bacterial and host cell surface components. In the case of *Salmonella* and *Shigella*, invasion is a multifactorial process (Nhieu and Sansonetti, 1999) which involves the translocation of different bacterial factors directly into the host cell by specific transport systems. In contrast, entry of *Yersinia* and *Listeria* into non-phagocytic cells implies direct interaction between a bacterial ligand and a mammalian receptor (Finlay and Cossart, 1997; Kuhn and Goebel, 2000). For *Yersinia*, invasion is mediated by the outer membrane protein Invasin upon binding to β_1 integrin receptors (Isberg and Leong, 1990). A similar strategy was developed by *L. monocytogenes* to enter mammalian cells.
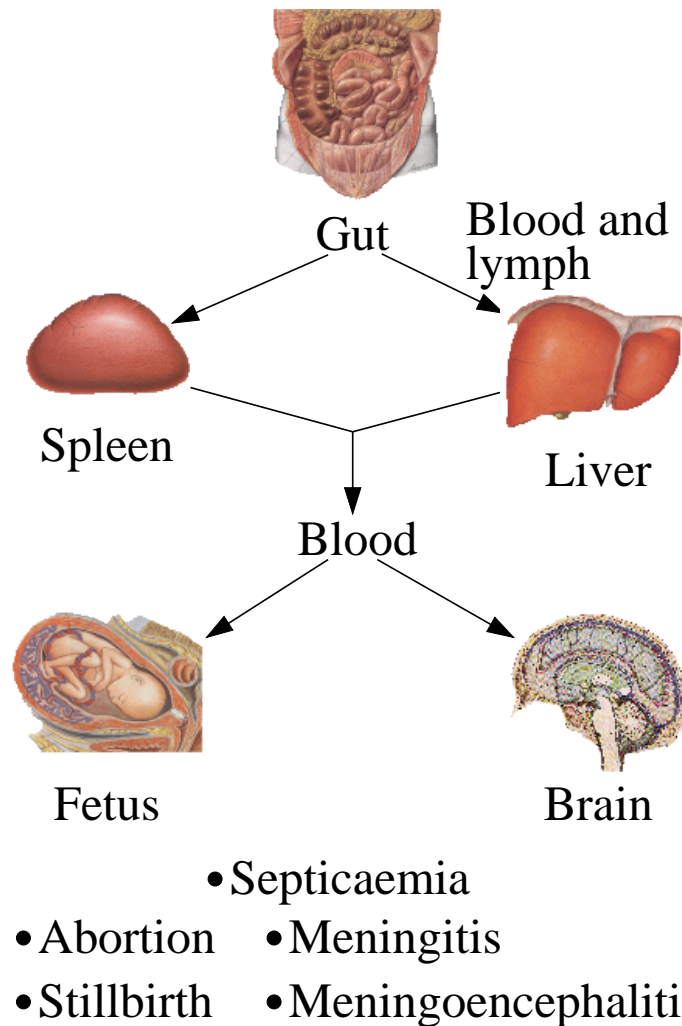
Figure 1.1: Overview of the course of human listeriosis.

To date, the role of two surface proteins involved in bacterial invasion of *L. monocytogenes* into non-phagocytic cells has been elucidated in detail at the molecular level, namely internalin (InlA) and InlB (recently reviewed by Kuhn and Goebel, 2000). These proteins were identified by screening a library of transposon mutants of *L. monocytogenes* for defective internalization into epithelial cells *in vitro*, hence the name internalin (Gaillard *et al*., 1991). Transposon insertion occurred in a chromosomal region containing two highly homologous genes, termed *inlA* and *inlB*. These genes are located adjacent to each other and constitute the *inlAB* operon.

InlA mediates internalization of *L. monocytogenes* into cells of the human enterocyte-like epithelial cell line Caco-2 by binding to E-cadherin, a calcium-dependent cell-cell adhesion molecule localized at the basolateral side of polarized epithelial cells (Gaillard *et al*., 1991; Mengaud *et al*., 1996; Hermiston and Gordon, 1995). However, an apical invasion of polarized Caco-2 cells was also observed involving formation of cellular microvilli which interact with the bacteria (Karunasagar *et al*., 1994). InlA-mediated entry

of *L. monocytogenes* occurs via the zipper mechanism, which is also used by *Yersinia* to invade non-phagocytic cells (Mengaud *et al.*, 1996; Swanson and Baer, 1995). In contrast to the trigger mechanism used by *Salmonella* and *Shigella* (Swanson and Baer, 1995; Francis *et al.*, 1993), in the zipper mechanism bacteria are progressively engulfed by the host cells in pocket-like structures without membrane ruffling. Expression of human E-cadherin in transfected mouse fibroblasts renders these cells permissive for invasion of *L. monocytogenes* (Mengaud *et al.*, 1996). Also, the normally non-invasive species *L. innocua* transformed with the *inlA* gene and InlA-coated latex beads are invasive for cells expressing E-cadherin (Gaillard *et al.*, 1991; Mengaud *et al.*, 1996; Lecuit *et al.*, 1997). For a long time the role of InlA *in vivo* was unclear, since an *inlAB* mutant was shown to reach the mesenteric lymph nodes and the liver as rapidly as the wild type strain after oral inoculation of mice (Dramsi *et al.*, 1995). However, a recent work reported that murine E-cadherin does not interact with InlA, excluding the mouse as a model for addressing the InlA function *in vivo* (Lecuit *et al.*, 2001).

In contrast to InlA, whose host cell spectrum is mostly restricted to epithelial cells, InlB promotes invasion into a wide variety of cell types *in vitro*, such as hepatocytes, endothelial cells and also some epithelial cells (Dramsi *et al.*, 1995; Ireton *et al.*, 1996; Parida *et al.*, 1998; Greiffenberg *et al.*, 1998). InlB-mediated entry into mammalian cells stimulates tyrosine phosphorylation of host cell adaptor proteins such as Gab1, Cbl and Shc, activation of the phosphoinositide (PI) 3-kinase p85-p110 and rearrangements in the actin cytoskeleton (Ireton *et al.*, 1996; Ireton *et al.*, 1999). Two different molecules were recently described as host cell receptors of InlB, namely gC1q-R, the ubiquitous receptor of the globular part of the complement component C1q (Braun *et al.*, 2000) and Met, a receptor tyrosine kinase whose only known ligand was the hepatocyte growth factor HGF, also known as scatter factor (Shen *et al.*, 2000). Interaction of InlB with both receptors was shown to mediate internalization of *L. monocytogenes* into non-phagocytic mammalian cells, which also occurs via the zipper mechanism. As shown for InlA, *L. innocua* expressing *inlB* and InlB-coated latex beads are also invasive for mammalian cells (Braun *et al.*, 1998), suggesting that each of these proteins is sufficient for entry into permissive cells. While the importance of InlA in virulence of *L. monocytogenes* could not be demonstrated in the mouse model, InlB was shown to play an important role in the hepatic phase of murine listeriosis by mediating bacterial invasion and/or replication in hepatocytes *in vivo* (Dramsi *et al.*, 1995; Gaillard *et al.*, 1996; Gregory *et al.*, 1997).

InlA is a 800 amino acid large surface-bound protein. As shown in Fig. 1.2, its primary structure contains a signal peptide, two repeat regions termed A and B, and a carboxy-terminal region with a putative cell wall anchor sequence (Gaillard *et al.*, 1991). Region A consists of 14 successive leucine-rich repeats (LRRs) of 22 amino acids each. Region B is made of three repeats of 70, 70 and 49 amino acids, respectively. The C-terminal region of InlA contains the pentapeptide LPTTG, followed by a stretch of hydrophobic amino acids spanning the cell membrane and a short tail of positively charged residues (Gaillard *et al.*, 1991; Lebrun *et al.*, 1996). The pentapeptide LPTTG corresponds to the consensus sequence LPXTG (where X is any amino acid) found in protein A of *Staphylococcus aureus* and other surface proteins of Gram-positive cocci (Fischetti *et al.*, 1990). This sequence is required for anchoring of surface proteins to the cell wall via covalent linkage between the carboxylic group of the threonine of the pentapeptide and a free amino group of the peptidoglycan (reviewed by Navarre and Schneewind, 1999).
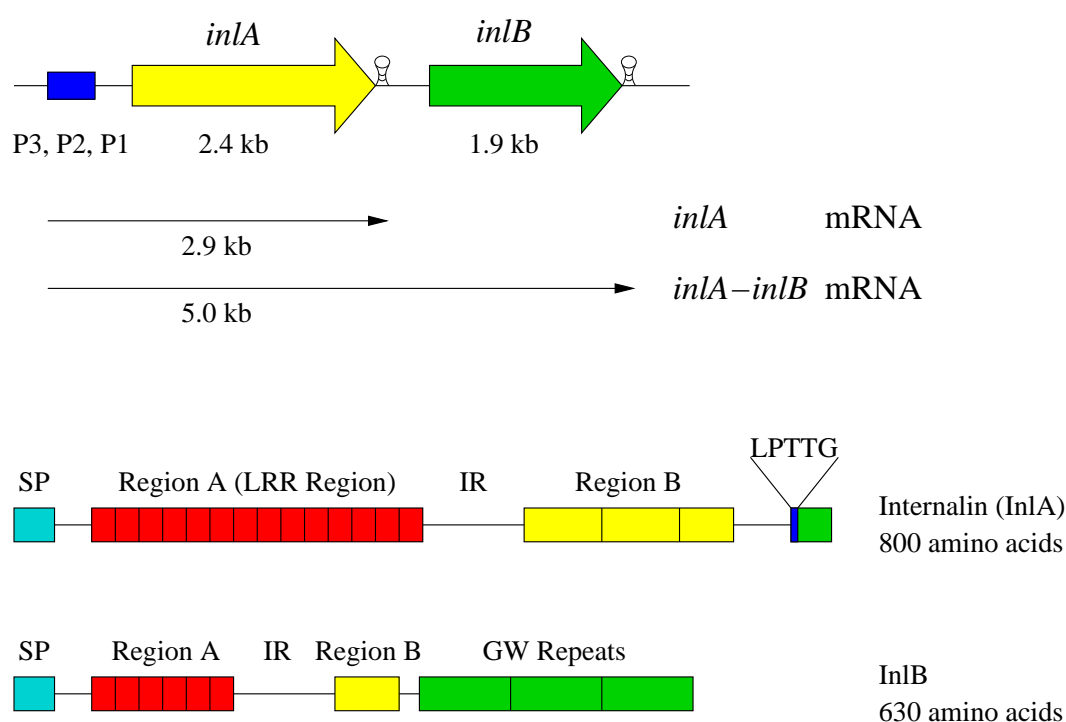
Figure 1.2: The *inlAB* operon and its derived proteins internalin (InlA) and InlB, based on Gaillard *et al.* (1991), Dramsi *et al.* (1993) and Braun *et al.* (1997). Promoters are indicated as P3, P2 and P1. Coding regions of the genes are shown as arrows. Putative transcriptional terminators are represented as stem-loop structures ( ). SP and IR indicate signal peptide and inter-repeat region, respectively.

InlB is a 630 amino acid surface protein which has six LRRs similar to those of InlA, but only one repeat of 56 amino acids in region B (Fig. 1.2). Interestingly, InlB is not only associated with the bacterial cell surface but also found in culture supernatants, indicating that a fraction of this protein is secreted or released from the bacterial cell (Lingnau *et al.*, 1995; Braun *et al.*, 1997; Jonquieres *et al.*, 1999). The loose association of InlB to the bacterial surface is mediated by the GW modules, which are three repeats of 80 amino acids beginning with the dipeptide glycine-tryptophan located at the 232 amino acid C-terminus of InlB (Braun *et al.*, 1997; Jonquieres *et al.*, 1999). The GW modules bind to lipoteichoic acids of the cell membrane, thus mediating association of InlB to the bacterial surface (Jonquieres *et al.*, 1999). Therefore, InlB shows a mechanism of protein association different from the LPXTG anchor motif.

LRR is a motif typical for the superfamily of leucine-rich repeat proteins with a defined periodicity of spaced leucine or isoleucine residues displaying the characteristic consensus sequence - L - - L - L - - N - L - D I - - L - - L -, whereas - represents any amino acid (Kobe and Deisenhofer, 1994). LRRs correspond to β–α structural units consisting of a short β-strand and an opposing antiparallel α-helix connected to each other by coils (Marino *et al.*, 2000). These structures result in a non-globular, horseshoe-shaped molecule. LRR domains are found mostly in eukaryotes in a variety of proteins with

different functions and cellular locations such as adhesive proteins, components of signal transduction cascades, proteoglycans of the extracellular matrix and products of plant disease resistance genes. The few examples of LRR proteins in prokaryotes include, in addition to the listerial internalins, the virulence factors YopM of *Yersinia pestis* (Leung and Straley, 1989), IpaH of *Shigella flexneri* (Hartman *et al.*, 1990) and SspH of *Salmonella typhimurium* (Miao *et al.*, 1999). The members of the LRR protein superfamily have in common their involvement in protein-protein interactions, in which binding of proteins to their ligands seems to be mediated by the LRR region. Indeed, it was shown that the LRR region of both InlA and InlB is necessary to promote bacterial entry into non-phagocytic mammalian cells by interacting with the corresponding cellular receptors (Lecuit *et al.*, 1997; Braun *et al.*, 1999).

The internalin genes *inlA* and *inlB* belong to a growing family of related virulence-associated genes which is known as the listerial internalin multigene family. Members of this family have been identified so far in *L. monocytogenes* and *L. ivanovii*, the other pathogenic species of the genus *Listeria* (Gaillard *et al.*, 1991; Engelbrecht *et al.*, 1996; Domann *et al.*, 1997; Dramsi *et al.*, 1997; Raffelsbauer *et al.*, 1998; Engelbrecht *et al.*, 1998a; Engelbrecht *et al.*, 1998b; Dominguez-Bernal, 2001). Recent studies have indicated that there are two classes of internalins: those representing relatively large proteins (> 50 KDa) which are mainly cell surface-associated, and those that are considerably smaller (≤ 30 KDa) and secreted by the bacterial cell. Both classes share highly homologous LRR units of varying numbers, possess in addition LRR-flanking sequences of considerable similarities and N-terminal signal sequences. The main difference between large and small internalins is the absence in the latter ones of the C-terminal part of the molecules comprising the B region and the cell wall anchor. Due to the lack of sequences that can anchor the proteins to the bacterial cell surface, these internalins are found exclusively in the culture supernatant. While InlC is the only small internalin characterized so far in *L. monocytogenes* (Engelbrecht *et al.*, 1996), four members of this class of internalins, namely i-InlC, i-InlD, i-InlE and i-InlF, were previously described in *L. ivanovii* (Engelbrecht *et al.*, 1998a; Engelbrecht *et al.*, 1998b). In addition, six new small internalin genes (i-*inlG*, i-*inlH*, i-*inlI*, i-*inlJ*, i-*inlK* and i-*inlL*) were recently detected in this *Listeria* species (Dominguez-Bernal, 2001). The function of the small internalins remains unclear but *in vivo* studies using the mouse model show that they represent important virulence factors (Engelbrecht *et al.*, 1996; Engelbrecht *et al.*, 1998b).

In addition to InlA and InlB, two other proteins, p60 and ActA, seem to be involved in internalization of *L. monocytogenes* into mammalian cells. The protein p60, encoded by the *iap* (invasion associated protein) gene, seems to mediate entry of *L. monocytogenes* into murine fibroblasts of the cell line 3T6 (Kuhn and Goebel, 1989). P60 has also a second function as murein hydrolase involved in septum separation during bacterial cell division (Wuenscher *et al.*, 1993). ActA is responsible for actin-based bacterial motility and cell-to-cell spread (Domann *et al.*, 1992; Kocks *et al.*, 1992). This large surface-bound protein also plays a role in adhesion and invasion of *L. monocytogenes* into mammalian cells by binding to heparan sulfate proteoglycans on the cell surface (Alvarez-Dominguez *et al.*, 1997).

After internalization bacteria undergo a characteristic intracellular replication cycle as shown in Fig. 1.3 comprising lysis of the phagosomal membrane, replication, intracellu-

lar movement and spread to adjacent cells (Tilney and Portnoy, 1989). Following entry bacteria first reside for approx. 30 min within the membrane-bound phagosomal vacuole, which is subsequently lysed allowing the microorganisms to escape into the cytoplasm. Bacteria that remain in the phagosome are killed and digested. Once free in the cytoplasm, listeriae begin to replicate with a doubling time of approx. 1h and concomitantly induce the nucleation of host actin filaments which form a cloud around the bacterial cell. These filaments are rearranged within 2 h into polar comet-like tails consisting of short actin filaments and other actin binding proteins. Formation of these actin tails at one pole of the bacteria is the propulsive force that bacteria use to move through the cytoplasm at a speed of approx. 0.3 μm/s. When moving bacteria contact the host cell plasma membrane, they induce the formation of pseudopode-like structures with the bacterium at the tip and the actin tail behind. Contact between these bacterium-containing protrusions and neighboring cells results in the phagocytosis of the protrusion. In the newly infected cell, the bacterium is surrounded by two plasma membranes which must be lysed to initiate a new cycle of replication and movement.
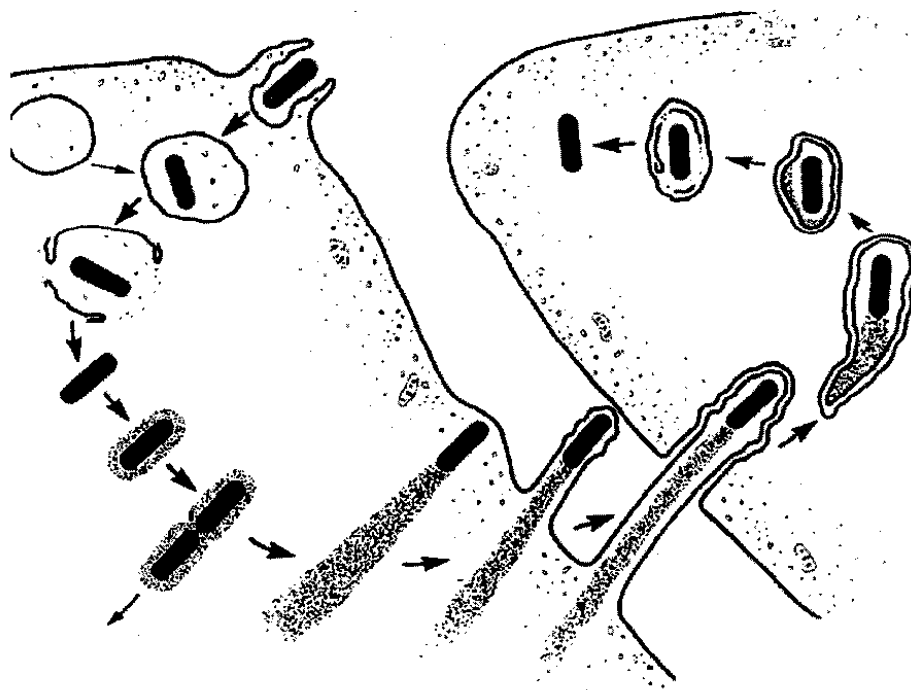
Figure 1.3: The intracellular replication cycle of *Listeria monocytogenes* (adapted from Tilney and Portnoy, 1989).

The events of the listerial intracellular replication cycle are mainly mediated by products of six genes, namely *prfA*, *plcA*, *hly*, *mpl*, *actA* and *plcB*, which are grouped together on a 10 kb region of the chromosome building the so-called PrfA-dependent virulence gene cluster of *L. monocytogenes* shown in Fig. 1.4 (for a review see Portnoy *et al.*, 1992). This cluster, which comprises in addition three small ORFs of unknown function

downstream of *plcB*, is flanked by two house-keeping genes: the *prs* gene encoding a
phosphoribosyl-pyrophosphate synthetase at the 5' side and the *ldh* gene coding for a lac-
tate dehydrogenase at the 3' side. The virulence gene cluster is also present in *L. ivanovii*
(Gouin *et al*., 1994; Lampidis *et al*., 1994), the animal pathogenic *Listeria* species which
displays a similar intracellular replication cycle as *L. monocytogenes* (Tilney and Portnoy,
1989; Karunasagar *et al*., 1993). The products of the virulence genes are listeriolysin, the
phospholipases PlcA and PlcB, a metalloprotease, the surface protein ActA and the tran-
scriptional activator PrfA.
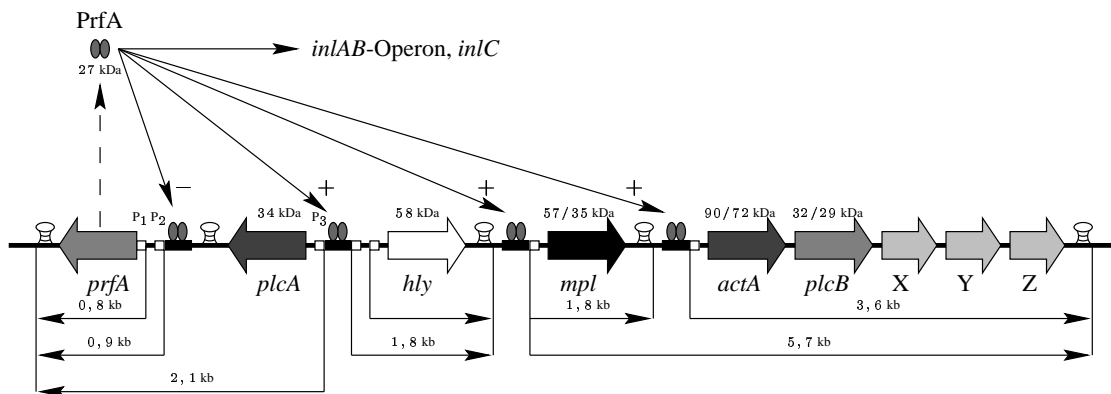
Figure 1.4: The PrfA-dependent virulence gene cluster of *L. monocytogenes*, adapted
from Engelbrecht (1999).

Listeriolysin O (LLO) is a pore-forming cytolysin encoded by the *hly* gene which me-
diates lysis of the phagosomal membrane, enabling the bacteria to escape from the phago-
some into the cytoplasm (Tilney and Portnoy, 1989). Due to its action, *L. monocytogenes*
produces characteristic zones of lysis when grown on blood agar plates. Hemolysis was
shown to be essential for virulence, since all pathogenic clinical isolates are hemolytic
and non-hemolytic strains are avirulent. Listeriolysin is a secreted protein of 58-60 KDa.
It is sulfhydryl-activated and belongs to a family of pore-forming cytolysins for which
streptolysin O of *Streptococcus pyogenes* is the prototype (Cossart *et al*., 1989). These
cytolysins are inhibited by low concentrations of cholesterol and oxygen and activated by
reducing agents. The optimal activity of listeriolysin is found at pH 5.5, which is in line
with its function in the acidified phagosome (Alouf and Geoffroy, 1991). When tested in
cellular *in vitro* assays, non-hemolytic *L. monocytogenes* mutants are as invasive as the
wild type strain, indicating that listeriolysin is not required for the listerial uptake into
non-phagocytic cells (Kuhn *et al*., 1988). However, these mutants are not able to repli-
cate within several non-phagocytic and phagocytic mammalian cells (Portnoy *et al*., 1988;
Kuhn *et al*., 1988). The *hly* gene is transcribed monocistronically from promoters located
in the intergenic region between *hly* and *plcA*. Apart from its role in promoting escape
from the vacuole, LLO is also able to induce eukaryotic cell death by apoptosis (Guzmán
et al., 1996), stimulate MAP kinases (Tang *et al*., 1996) and contribute to induction of
expression of cell-adhesion molecules in infected endothelial cells (Drevetts, 1997).

Lysis of the phagosomal membrane involves also the participation of PlcA, a 34 KDa phosphatidylinositol-specific phospholipase (PI-PLC) encoded by the *plcA* gene (Camilli *et al.*, 1993). PlcA has a broad pH spectrum ranging from 5.5 to 7.0. The *plcA* gene is transcribed together with the *prfA* gene into a bicistronic transcript. Deletion of *plcA* affects the ability of *L. monocytogenes* to escape from the phagosome, but results only in a slight decrease in virulence. PlcA seems to act synergetically with listeriolysin within the acidified phagosomal vacuole of the host cell to mediate lysis of the vacuolar membrane. However, PlcA alone is not able to promote bacterial release.

In addition to PlcA (PI-PLC), *L. monocytogenes* produces a second phospholipase C encoded by the *plcB* gene which specifically hydrolyzes phosphatidylcholine (lecithin). This lecithinase, termed PlcB or PC-PLC for phosphatidylcholin-specific phospholipase C, is a zinc-dependent enzyme with pH optimum between 6 and 7. The gene *plcB* is part of an operon which includes in addition the genes *mpl*, *actA* and the three small ORFs X, Y and Z. The deduced PlcB protein has a molecular weight of 32 KDa and is inactive until its maturation into a 29 KDa active enzyme which is accomplished by the metalloprotease encoded by the *mpl* gene (Poyart *et al.*, 1993). Interestingly, *plcB* mutants are unable to lyse the double membrane of the vacuole which is formed during cell-to-cell spread of *L. monocytogenes*, but they are fully invasive and replicate and move within the host cell as efficiently as the wild type strain (Vazquez-Boland *et al.*, 1992; Raveneau *et al.*, 1992).

As mentioned above, *L. monocytogenes* is able to induce nucleation of host actin filaments and formation of actin tails which are used by the bacteria to move in the host cell cytosol. These processes are mediated by the *actA* gene product (Domann *et al.*, 1992; Kocks *et al.*, 1992). ActA is a proline-rich protein of 610 amino acids anchored in the bacterial surface by a hydrophobic region at the C-terminus which acts as membrane anchor. Mutations in *actA* result in the loss of virulence, in the lack of intracellular actin polymerization and in the inability of intracellular movement. Inside the host cells, the *actA* mutant forms microcolonies located near the nucleus (Domann *et al.*, 1992). The internal proline-rich repeats are essential for the actin nucleation activity of ActA (Pistor *et al.*, 1994). Various host cell proteins were found associated with the actin tails, such as profilin, VASP, the Arp2/Arp3 complex, α-actinin, tropomyosin, vinculin and talin (reviewed by Ireton and Cossart, 1997). VASP was shown to bind purified ActA and profilin *in vitro*, thus establishing the link between ActA and the cytoskeleton (Chakraborty *et al.*, 1995). Phosphorylation of ActA within host cells seems to be an important step in modulation of the intracellular activity of ActA (Brundage *et al.*, 1993). Actin tail formation and cell-to-cell spread has also been reported in *L. ivanovii* by an ActA-related protein (Kreft *et al.*, 1995b; Karunasagar *et al.*, 1993) and in *Shigella flexneri* by IcsA, an ActA-analog protein encoded by the *icsA* gene, which is also called *virG* (Bernardini *et al.*, 1989).

The genes of the virulence gene cluster are regulated by the transcriptional activator PrfA, a cytoplasmic protein of 27 KDa encoded by the *prfA* (positive regulatory factor A) gene, which is part of the virulence gene cluster (Leimeister-Wächter *et al.*, 1990). A *prfA*-like gene is also present in *L. ivanovii*, where it regulates virulence genes similar to those of *L. monocytogenes* (Lampidis *et al.*, 1994). PrfA is a member of the Crp/Fnr family of prokaryotic transcriptional regulators which have been detected mainly in Gram-negative bacteria (Lampidis *et al.*, 1994; Kreft *et al.*, 1995a), and shares con-

served functional domains with Crp. In its C-terminal part, PrfA contains a conserved helix-turn-helix motif also present in other members of the Crp/Fnr family. In addition, PrfA carries a leucine zipper and a second helix-turn-helix motif at its N-terminus. The *prfA* gene is transcribed both monocistronically and bicistronically together with the *plcA* gene (Freitag *et al*., 1993). PrfA regulates its own transcription and that of the other virulence genes by a very complex mechanism. Under some culture conditions, PrfA can be present but inactive, which suggests the involvement of a cofactor or post-translational modifications for fully activity (Renzoni *et al*., 1997). Recent evidences indicate the existence of such a PrfA-activating factor, termed Paf (Böckmann *et al*., 1996; Dickneite *et al*., 1998) and that PrfA is upregulated during interaction of *L. monocytogenes* with mammalian cells and eukaryotic cell extracts (Renzoni *et al*., 1999). PrfA activates transcription of most virulence genes by binding to a 14-bp palindromic consensus sequence, termed "PrfA-box", which is centered at about position -40 from the transcriptional start site (Freitag *et al*., 1993; Sheehan *et al*., 1995). Mismatches within this sequence reduce binding affinity and transcription activation. Transcription of virulence genes is also influenced by temperature and nutrients. It was shown that expression of PrfA-regulated genes is low when bacteria are grown in rich culture media such as BHI (brain-heart infusion), but can be induced when bacteria are shifted from this broth into MEM, a minimum essential medium (Sokolovic *et al*., 1993; Bohne *et al*., 1994).

As shown in Fig. 1.2, the *inlAB* operon is transcribed into two transcripts: a 5,000-nt long transcript which spans both *inlA* and *inlB* genes and a smaller 2,900-nt one that covers only *inlA* (Dramsi *et al*., 1995). Transcription of these genes is only in part controlled by PrfA and is rather repressed in MEM (Dramsi *et al*., 1993; Lingnau *et al*., 1995; Bohne *et al*., 1996) . In contrast, the small internalin genes seem to be under strict PrfA control and are up-regulated after a MEM shift, similarly to the other known PrfA-dependent virulence genes (Engelbrecht *et al*., 1996; Engelbrecht *et al*., 1998b). Furthermore, there is evidence that expression of the small internalin genes may be induced during intracellular replication of the bacteria, in contrast to the large internalins which are better expressed extracellularly (Engelbrecht *et al*., 1996; Dramsi *et al*., 1993; Dickneite *et al*., 1998).

Aims of this study

In a previous work (Raffelsbauer, 1997) a gene locus containing internalin-related genes was detected on the chromosome of *L. monocytogenes* EGD. The aims of the present study were to identify and characterize these internalin genes in detail, determine their nucleotide sequences, study their occurrence in other *L. monocytogenes* serotypes, characterize their expression patterns and, in collaboration with B. Bergmann (doctoral thesis in progress), study their function *in vitro* and *in vivo* by constructing isogenic in-frame deletion mutants lacking these genes individually or in combination with other known internalin genes.

Results

Invasion of the pathogenic facultative intracellular bacterium *Listeria monocytogenes* into non-phagocytic mammalian cells *in vitro* was shown to depend on the internalins InlA and InlB (Gaillard *et al*., 1991; Dramsi *et al*., 1995). However, deletion of the *inlAB* gene cluster did not reveal a significant effect on the bacterial virulence in mice (Dramsi *et al*., 1997; Dramsi *et al*., 1995), suggesting that the presence of these proteins *in vivo* is not essential for pathogenesis or might be circumvented by other virulence factors. Early studies revealed by Southern blot hybridization the presence of other related internalin genes on the chromosome of *L. monocytogenes* in addition to the *inlA* and *inlB* genes and indicated the existence of an internalin multigene family (Gaillard *et al*., 1991). Later, a third member of this family, termed *inlC*, was identified using different strategies (Engelbrecht *et al*., 1996; Domann *et al*., 1997). InlC seems to be an important virulence factor of *L. monocytogenes*, since the absence of this protein leads to an increased LD_{50} in the mouse model (Engelbrecht *et al*., 1996).

Previously, a chromosomal fragment termed lisM51 showing 60% identity to the *inlA* gene was detected in the clinical isolate *L. monocytogenes* JBL 1231 (serotype 4b) applying a subtracter probe hybridization method to recover *L. monocytogenes*-specific virulence sequences (Chen *et al*., 1993). In a previous study (Raffelsbauer, 1997), a DNA fragment was amplified from the chromosome of *L. monocytogenes* EGD by polymerase chain reaction (PCR) using oligonucleotides derived from the lisM51 sequence[1]. Nucleotide sequencing of this PCR product revealed 92% identity to the fragment derived from *L. monocytogenes* JBL 1231, indicating the presence of this chromosomal region in strain EGD, which belongs to the serotype 1/2a. However, no product was obtained by performing the same PCR using chromosomal DNA isolated from the closely related, other pathogenic *Listeria* species *L. ivanovii* strain ATCC 19119. The lisM51 sequence amplified from *L. monocytogenes* EGD contained an open reading frame (ORF) with high identity to *inlA* and represented therefore a putative new internalin gene, which was termed *inlY*. Using oligonucleotides derived from this gene, successive inverse PCRs were performed in both 5' and 3' directions. Nucleotide sequencing of the obtained PCR products detected the presence of further internalin genes located up- and downstream from *inlY*, which were designated as *inlX* and *inlZ*, respectively (Raffelsbauer, 1997).

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[1]The nucleotide sequence of lisM51 was deposited in the database GenEMBL under the accession number L16018.

2.1   Identification and characterization of new internalin genes of *Listeria monocytogenes* EGD

In the present study, the chromosomal locus containing the putative new internalin genes detected previously (Raffelsbauer, 1997) was examined in more detail. In order to verify and complete the existing nucleotide sequence data, a chromosomal region of 6,786 bp comprising the entire gene locus and its flanking genes was amplified by PCR using chromosomal DNA isolated from *L. monocytogenes* EGD. This region was divided into five overlapping fragments, which were amplified in three independent reactions each. The obtained PCR products were cloned into the vector pUC18 and individually sequenced, yielding nucleotide sequence data of approx. 21 kb in length. The complete nucleotide sequence of the *inlGHE* gene cluster and its flanking genes is shown in App. B.1 and is available in the EMBL nucleic acid database under the accession number AJ007319.

2.1.1   The new internalin gene cluster *inlGHE*

As shown in Fig. 2.1, the chromosomal region of *L. monocytogenes* EGD characterized in the present study carries a gene cluster containing three new internalin genes. This cluster is flanked by two listerial house-keeping genes coding for proteins with homology to the 6-phospho-β-glucosidase and the succinyl-diaminopimelate desuccinylase (69% and 26% identity to the enzymes of *E. coli*, respectively). These genes were termed *ascB* and *dapE* in analogy to the corresponding *E. coli* genes. While this study was in progress a similar gene cluster was reported also showing three new internalin genes, termed *inlC2*, *inlD* and *inlE*, which were flanked by the same house-keeping genes (Dramsi *et al.*, 1997). The sequences of two (*inlC2* and *inlD*) of the three reported *inl* genes were, however, different from those identified here. A comparison of the complete sequence of the two gene clusters revealed interesting findings: first, there is another ORF downstream from the *ascB* gene encoding InlG, a new large internalin different from the hitherto described large internalins. Secondly, the *inl* gene cluster identified in the present study does not contain the previously reported *inlC2* and *inlD* genes but instead a new one, *inlH*, which is apparently generated by an in-frame deletion removing the 3'-terminal part of *inlC2* and the 5'-terminal part of *inlD*. The deletion seems to be generated by recombination between the almost identical first repeat units of the B region of *inlC2* and *inlD*. A comparison of the new *inlGHE* gene cluster to the *inlC2DE* locus showing the deletion site and an amino acid sequence alignment of the proteins InlC2, InlD and InlH are depicted in Fig. 2.2.

The here identified genes *inlG*, *inlH* and *inlE* are preceeded by potential ribosome binding sites (AAGGAG) located 8, 7 and 8 bp upstream from the start codon ATG, respectively. Putative transcription terminators were detected 11, 11 and 9 bp downstream from the stop codons TAA, suggesting a monocistronic transcription of the genes of the *inlGHE* cluster. Intergenic regions were of 137 and 207 bp between *inlG* and *inlH* and between *inlH* and *inlE*, respectively. Intergenic regions between the *inlGHE* cluster and the flanking genes *ascB* and *dapE* were of 273 and 134 bp, respectively.

Most virulence genes of *L. monocytogenes*, including internalin genes, are regulated
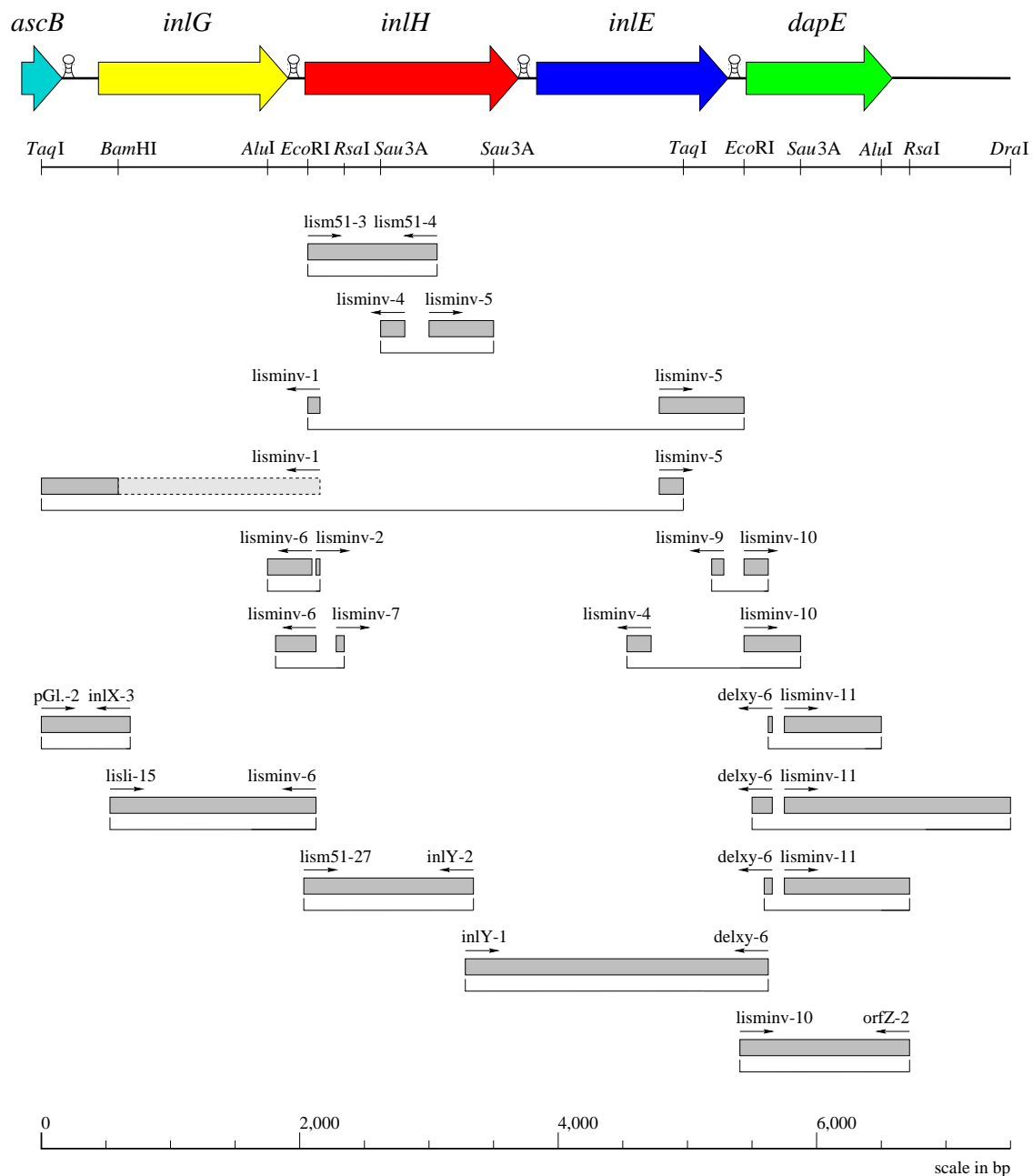
Figure 2.1: Genetic organization of the *inlGHE* gene cluster of *L. monocytogenes*. The strategy used for molecular cloning and nucleotide sequencing of the genes *inlG*, *inlH* and *inlE*, and the flanking house-keeping genes *ascB* and *dapE* is shown. Coding regions of the five genes are depicted as arrows. Putative transcriptional terminators are indicated as stem-loop structures ( ). Target sites of restriction enzymes used to prepare template DNA for inverse PCRs are indicated. Used oligonucleotides and obtained PCR products are shown. The Figure also contains PCR products already shown in Raffelsbauer (1997). The DNA fragment amplified with the primers lism51-3 and lism51-4 (top) corresponds to the lisM51 sequence. All PCR products were cloned into the vector pUC18 and sequenced. The nucleotide sequence of the *inlGHE* gene cluster, including the 3' end of *ascB* and the *dapE* gene, was deposited in the EMBL database under the accession number AJ007319. Figure in scale.

A

```
     BESTFIT of: inlGHE  check: 5678  from: 1  to: 6786
      to: inlC2DE  check: 1127  from: 1  to: 5746
LOCUS        LMU77368   5746 bp  DNA        BCT      29-AUG-1997
DEFINITION   Listeria monocytogenes internalin (inlC2), inlD,
    and inlE genes, complete cds.
ACCESSION    U77368
NID          g2347103
     Gap Weight:       50     Average Match: 10.000
  Length Weight:        3     Average Mismatch: -9.000
        Quality:    32118             Length:    5746
          Ratio:    8.379               Gaps:       3
Percent Similarity: 99.556   Percent Identity: 99.556
      Match display thresholds for the alignment(s):
                        | = IDENTITY
                        : =    5
                        . =    1

             .         .         .         .         .
 3008 CAATCAGTCACTTTCAAAAATACAACAGTTCCGTTTAGTGGGACAGTTAC 3057
      |||||||||||||||||||||||||||||||||||||||||||||||||
 1201 CAATCAGTCACTTTCAAAAATACAACAGTTCCGTTTAGTGGGACAGTTAC 1250

             .         .         .         .         .
 3058 ACAACCATTAACAGAAGCTTACACTGCGGTTTTTGACGTGGACGGGAAGC 3107
      |||||||||||||||||||||||||||||||||||||||||||||||||
 1251 ACAACCATTAACAGAAGCTTACACTGCGGTTTTTGACGTGGACGGGAAGC 1300

             .         .         .         .         .
 3108 AAACAAGTGTGACAGTCGGCGCGAATGAATTAATTAAAGAACCAACGGCC 3157
      |||||||||||||||||||||||||||||||||||||||||||||||||
 1301 AAACAAGTGTGACAGTCGGCGCGAATGAATTAATTAAAGAACCAACGGCC 1350

             .         .         .         .         .
 3158 CCGACGAAAGAAGGT................................... 3172
      |||||||||||||||
 1351 CCGACGAAAGAAGGTTACACATTCACAGGCTGGTATGATGCAAAAACTGG 1400
                                                       .

                                                       .
                                                       .
             .         .         .         .         .
 3173 .........................TACACTTTCACAGGCTGGTATGAT 3196

 3251 AACCAATAGCACCAACCAAAGAAGGCTACACTTTCACAGGCTGGTATGAT 3300

             .         .         .         .         .
 3197 GCGAAAACTGGCGGAACTAAATGGGATTTTGCGACAGATAAAATGCCAGC 3246
      ||||||||||||||| ||||||||||||||||||||||||||||||||||
 3301 GCGAAAACTGGCGGAAATAAATGGGATTTTGCGACAGATAAAATGCCAGC 3350

             .         .         .         .         .
 3247 AGAGGACATCACATTATACGCGCAGTTTACGATTAATAGTTACACAGCCA 3296
      |||||||||||||||||||||||||||||||||||||||||||||||||
 3351 AGAGGACATCACATTATACGCGCAGTTTACGATTAATAGTTACACAGCCA 3400

             .         .         .         .         .
 3297 CATTTGATATTGATGGTAAATTAACGACTCAAAAAGTCACTTATCAAAGC 3346
      |||||||||||||||||||||||||||||||||||||||||||||||||
 3401 CATTTGATATTGATGGTAAATTAACGACTCAAAAAGTCACTTATCAAAGC 3450
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B

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            1                                                 50
InlC2   MKKRWNSVFK LVLMVTAILG LSLYVTTSQG VEVRAESITQ PTAINVIFPD
InlH    MKKRWNSVFK LVLMVTAILG LSLYVTTSQG VEVRAESITQ PTAINVIFPD
InlD    .MKRNKTALR ILVTLAVVMA ISFWVGTS.S KEVQAAEIGQ PTPINEIFTD
            51                                                100
InlC2   PALANAIKIA AGKSNVTDTV TQADLDGITT LSAFGTGVTT IEGVQYLNNL
InlH    PALANAIKIA AGKSNVTDTV TQADLDGITT LSAFGTGVTT IEGVQYLNNL
InlD    ENLANAIKTT LSKPSTASAV SQVELDSVRD VTAESSNIAS LEGVQYLNNL
            101                                               150
InlC2   IGLELKDNQI TDLTPLKNLT KITELELSGN PLKNVSAIAG LQSIKTLDLT
InlH    IGLELKDNQI TDLTPLKNLT KITELELSGN PLKNVSAIAG LQSIKTLDLT
InlD    DTLVLNNNKI TDLNPLAGLT KLSILEASNN QLSDISALSN VTNLHQLRLD
            151                                               200
InlC2   STQITDVTPL AGLSNLQVLY LDLNQITNIS PLAGLTNLQY LSIGNAQVSD
InlH    STQITDVTPL AGLSNLQVLY LDLNQITNIS PLAGLTNLQY LSIGNAQVSD
InlD    GNQIKQLNGV SNLINLETIE LSNNQITAIS PVSGLKNLVG LGIDNNKISD
            201                                               250
InlC2   LTPLANLSKL TTLKADDNKI SDISPLASLP NLIEVHLKNN QISDVSPLA.
InlH    LTPLANLSKL TTLKADDNKI SDISPLASLP NLIEVHLKNN QISDVSPLA.
InlD    LSPISGLSKL NHLTADSNQI SDLRPLSNLA AMEVMRLDGN QISDVTPIAN
            251                                               300
InlC2   .......... .......... ....NTSNLF IVTLTNQTIT NQPVFYQNNL
InlH    .......... .......... ....NTSNLF IVTLTNQTIT NQPVFYQNNL
InlD    LANLNYVFLA ENQISDISSL QPLFNSPNFF GITLDNQKIT SEPVLYQQEL
            301                                               350
InlC2   VVPNIVKGPS GAPIAPATIS DNGTYASPNL TWNLTSFINN VSYTFNQSVT
InlH    VVPNVVKGPS GAPIAPATIS DNGTYASPNL TWNLTSFINN VSYTFNQSVT
InlD    VVPNNIKDEM GALIAPATIS DNGVYASPNI NWNLPNYTNQ VSYTFNKQLA
            351                                               400
InlC2   FKNTTVPFSG TVTQPLTEAY TAVFDVDGKQ TSVTVGANEL IKEPTAPTKE
InlH    FKNTTVPFSG TVTQPLTEAY TAVFDVDGKQ TSVTVGANEL IKEPTAPTKE
InlD    Y....GSFSG TVTQPLHNAY TATFDVDGVK TNEAVEETKL LQEPIAPTKE
            401                                               450
InlC2   GYTFTGWYDA KTGGNKWDFG VDKMPAENIT LYAQFTINSY TASFDNDGKL
InlH    GYTFTGWYDA KTGGTKWDFA TDKMPAEDIT LYAQFTINSY TATFDIDGKL
InlD    GYTFTGWYDA KTGGNKWDFA TDKMPAEDIT LYAQFTINSY TATFDIDGKL
            451                                               500
InlC2   TTQKVTYQSL LEEPAAPTKT GYTFKGWYDA KTGGTKWDFA TGKMPAGNIT
InlH    TTQKVTYQSL LEEPVAPTKD GYTFTGWYDA KTGGTKWDFA TGKMPAGNIT
InlD    TTQKVTYQSL LEEPVAPTKD GYTFTGWYDA KTGGTKWDFA TGKMPAGNIT
            501                                               550
InlC2   LYAQFTKNDS PNPNDPTPNT PTGNGDGTSN PSNSGGNTTL PTAGDENTML
InlH    LYAQFTKNDN PNPDDPTTNT PTGNGDGTSN PSNSGGNTTL PTAGDENTML
InlD    LYAQFTKNDN PNPDDPTTNT PTGNGDGTSN PSNSGGNTTL PTAGDENTML
            551            574
InlC2   PIFIGVFLLG TATLILRKTI KVK*
InlH    PIFIGVFLLG TATLILRKTI KVK*
InlD    PIFIGVFLLG TATLILRKTI KVK*
```

Figure 2.2: A. Nucleotide sequence comparison of the gene clusters *inlGHE* (upper line) and *inlC2DE* (under line) created by the program BestFit (Genetics Computer Group, University of Wisconsin). Pipes indicate identical nucleotides. Dots represent gaps inserted to maximize the alignment. Nucleotide positions within the sequences are listed at both margins. Three dots in the middle represent a jump in the alignment due to the deletion of the 3' terminal end of *inlC2* and the 5' portion of *inlD* compared to the new *inlH* gene. B. Multiple alignment of the amino acid sequences of the proteins InlC2, InlH and InlD generated using the program pileup (HUSAR, German Cancer Research Center). As indicated by grey boxes, the amino acid sequence of InlH is identical to that of InlC2 in the first two-thirds of the molecule and identical to that of InlD at the C-terminus (except for one amino acid in both cases). Numbers above the sequence indicate the position of the corresponding amino acids.

by the transcriptional activator protein PrfA (Bohne *et al.*, 1996; Engelbrecht *et al.*, 1996). A computational search within the *inlGHE* gene cluster for promoter-like sequences containing the PrfA binding site TTAACANNTGTTAA was performed using the program findpatterns provided by the GCG software package (Genetics Computer Group, University of Wisconsin). The sequences TTAACANNTGTTAA(N){125,135}ATG and TTAA-CANNTGTTAA(N){20,30}TAANAT(N){100,110}ATG were used as patterns, whereas N is any nucleotide and the numbers in parenthesis represent an interval of the quantity of the previous nucleotide (N). This search yielded 111 finds, but only three of them were located in intergenic regions. A sequence was found which contains four mismatches compared to the perfect PrfA-box and is centered 62 base pairs upstream from the start codon of *inlH*. This distance is very short and also the putative imperfect Pribnow box is located very close to the start codon (-22 base pairs). Therefore, it is unlikely that this sequence serves as transcription start for the *inlH* gene. Another sequence containing three mismatches compared to the perfect palindrome and centered at position -111 from the start codon of *inlE* was also detected. This sequence is located at position -40 relative to a putative Pribnow box detected at position -10. A third sequence was found at position -80 from the start codon of the *dapE* gene which also contains four mismatches in comparison to the ideal PrfA box. However, this sequence is centered at position -30 relative to a putative imperfect Pribnow box.

2.1.2   Molecular cloning of the *inlGHE* gene cluster

Molecular cloning of the *inlGHE* gene cluster and its flanking genes was performed as summarized in Fig. 2.1. A 979-bp DNA fragment of *inlH* was amplified by direct PCR from the chromosome of *L. monocytogenes* EGD using the oligonucleotides lism51-3 and lism51-4 derived from the sequence lisM51 (GenEMBL, acc. number L16018). To extend the sequence downstream of this DNA fragment, inverse PCRs were performed using *Sau*3A- and *Eco*RI-cleaved and religated DNA as template and the primer pairs lisminv-4/lisminv-5[2] and lisminv-1/lisminv-5, respectively. The sequence upstream of *inlH* was obtained by inverse PCRs using as template *Taq*I, *Alu*I and *Rsa*I digested DNA and the primer combinations lisminv-1/lisminv-5, lisminv-6/lisminv-2 and lisminv-6/lisminv-7, respectively. The PCR product obtained with *Taq*I-digested DNA was cleaved with *Bam*HI. Only the fragment between the *Taq*I and *Bam*HI restriction sites was cloned and sequenced. The region downstream of *inlE* containing the *dapE* gene was extended by successive inverse PCRs using the restriction enzymes *Rsa*I, *Sau*3A, *Alu*I, *Dra*I and *Rsa*I and the primer pairs lisminv-9/lisminv-10, lisminv-4/lisminv-10, delxy-6/lisminv-11, delxy-6/lisminv-11 and delxy-6/lisminv-11, respectively. The complete DNA region containing the genes *ascB*, *inlG*, *inlH*, *inlE* and *dapE* was then divided into five overlapping fragments which were amplified by direct PCRs using the primer combinations pGluco-2/inlX-3, lisli-15/lisminv-6, lism51-27/inlY-2, inlY-1/delxy-6 and lisminv-10/orfZ-2. Three independent reactions were performed per primer combination, and the obtained PCR products were cloned into the vector pUC18 and sequenced.

---

[2]Primers lisminv-4 and lisminv-5 hybridized to both *inlH* and *inlE* genes.

2.2 The genes *inlG* and *inlH* are present in most *L. monocytogenes* serotypes

In order to test whether *inlG* is specific for strain EGD or is also present in other *L. monocytogenes* strains, PCRs were performed using *inlG*-specific primers and chromosomal DNA derived from several *L. monocytogenes* isolates belonging to different serotypes. As shown in Fig. 2.3, most *L. monocytogenes* strains tested yielded PCR products of the expected size, which is 1,001 bp (Scheinpflug, 1998). A similar PCR product was not obtained with chromosomal DNA isolated from the avirulent, closely related species *L. innocua*, suggesting the occurrence of *inlG* in *L. monocytogenes* but not in *L. innocua*. Using another primer pair that specifically probes for the presence of *inlC2* and *inlD* or *inlH*, which can be distinguished according to the length of the PCR product obtained (2,706 or 794 bp, respectively), *inlH* was detected in most *L. monocytogenes* serotypes tested (Fig. 2.3). Note that in the serotype 1/2b not the *inlG* but the *inlH* gene could be detected, whereas in serotype 4c PCR with *inlH*-specific primers failed to give a product, but the *inlG* gene is present. Only serotype 4d was negative for both *inlG* and *inlH*. Interestingly, the *inlC2DE* cluster could not be detected in any of the strains tested, suggesting that this cluster might be specific for the EGD strain used in Dramsi *et al.* (1997).

A schematic comparison of the *inlGHE* and *inlC2DE* gene clusters showing the hybridization sites of the used primers and the expected PCR products of the reactions performed is depicted in Fig. 2.3B. The chosen reaction conditions for amplification of *inlC2* and *inlD* or *inlH* allow the synthesis of products of 3 kb in length. PCRs were performed using chromosomal DNA which was previously tested by performing PCRs with serotype-specific primers (data not shown). The obtained products showed expected sizes and similar amounts (Scheinpflug, 1998). Therefore, differences in the amount of the PCR products obtained with *inl*-specific primers might reflect possible sequence variations within the hybridization sites of the used primers.

2.3 Sequence of the new internalins InlG, InlH and InlE

The proteins InlG, InlH and InlE display all features characteristic for the large, cell wall-bound internalins, whose prototype is represented by InlA: a signal sequence of 35 or 33 amino acids, two repeat regions (region A and region B) and a C-terminal cell wall anchor. The amino acid sequences of these three internalins are shown in Fig. 2.4. The principal features of the proteins such as signal peptides, putative cleavage sites of the signal peptidase 1, regions A, B and D, and cell wall anchor motives are highlighted.

As shown in Fig. 2.4, the new *inlG* gene encodes a protein of 490 amino acids, which contains in region A four consecutive leucine-rich repeat (LRR) units of 22 amino acids, based upon a definition[3] adapted from Kobe and Deisenhofer (1994). Region B is composed of two repeats of 70 and 49 amino acids, which are similar to the B repeats number

---

[3]To determine the number of leucine-rich repeats (LRRs), the consensus sequence - L - - L - L - - N - L - D I - - L - - L - was applied, in which leucine and isoleucine residues are regarded as interchangeable and - represents any amino acid. Variations within this sequence, e. g. exchange of (iso)leucine by valine were tolerated.
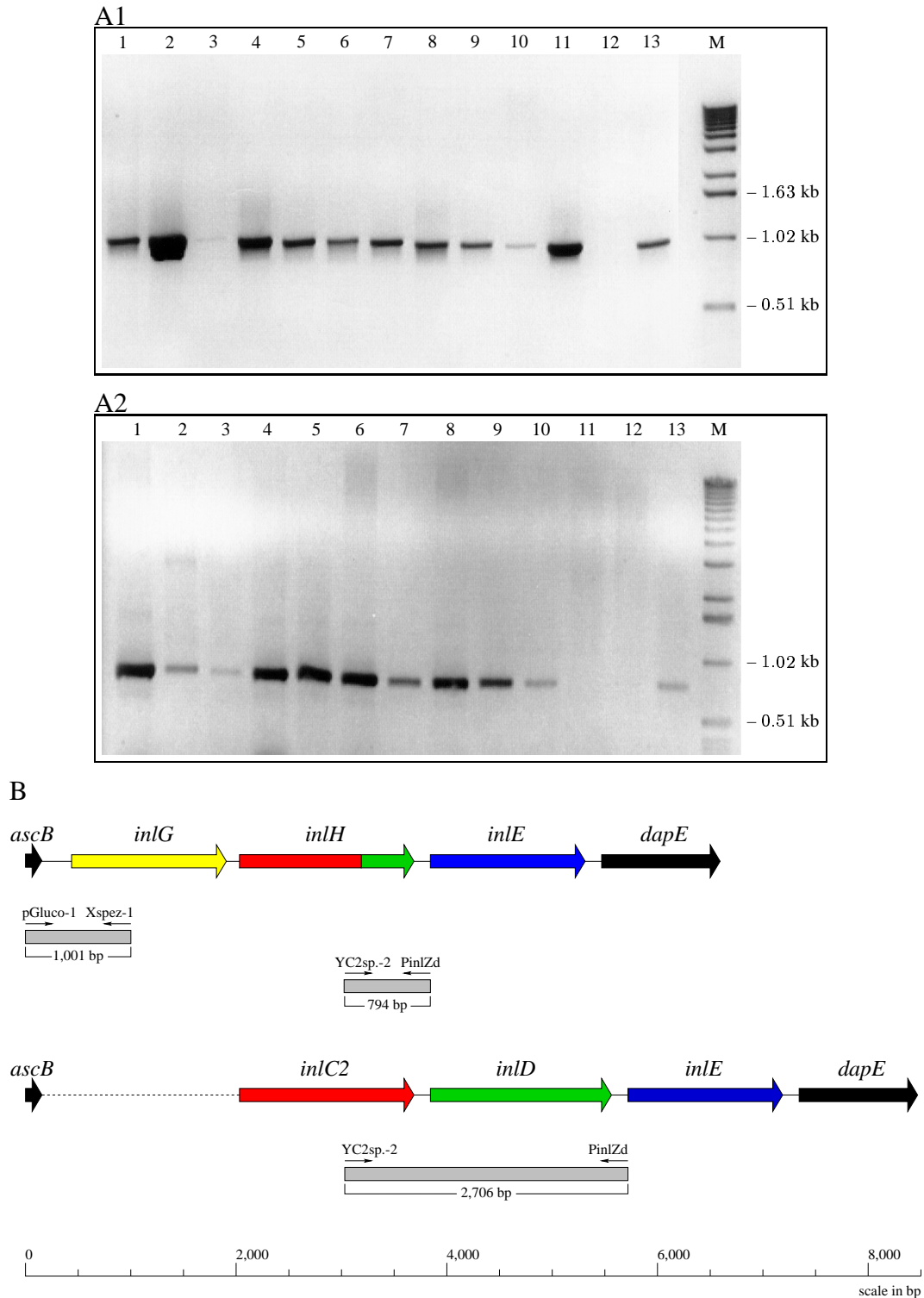
Figure 2.3: A. Detection of the genes *inlG* (A1) and *inlH* (A2) in different *L. monocytogenes* serotypes by PCR using the primer pairs pGluco-1/Xspez-1 and YC2spez-2/PinlZd, respectively. The hybridization sites of the primers and length of the expected PCR products are shown in B. Lanes: 1 – 13, *L. monocytogenes* serotypes 1/2a strain EGD (lane 1); 1/2a strain NCTC 7973 (2); 1/2 b (3); 1/2 c (4); 1/2 c strain LO28 (5); 3a (6); 3b (7); 3c (8); 4a (9); 4b (10); 4c (11); 4d (12) and 4e (13); M, DNA molecular weight standard. B. Schematic comparison of the *inlGHE* and *inlC2DE* gene clusters. The *inlH* gene is represented as chimeric gene generated by fusion of *inlC2* and *inlD*. PCRs performed to test for the presence of *inlG* and *inlH* (or alternatively *inlC2* and *inlD*) are indicated.

InlG (490 amino acids)

```
                                                        ↓
MKQRKTSVLHVLLVVTAILGISLWVNASHGMKAQAESIAQPAPINEIFT
DPALADEVKTELGKTSVTDEVTQTDLNQITKLEADDKGINSIEGIQYLT
NLNMLGVSSNQITNITPLANLT
NLDSLYLGDNKISDVTPLSGLT
QLTFVQLSINQIKDVTPLANLT
KLNYLDLRENQISDASPLVNMT
DLTVLHLEKQQITAAPVVYQTN
LVAPDILKNAYGEVVPPTTISNNGTFASPNITWNLDSFTSEVSYDFN
QKITLGDNGKVTFAGTVVQPIVEAPVNYITTFDVDGTTTTENVVVDT
LITEPAEPTKEGYTFSGWYDAETGGNEWDFAVDKMPATNMTLYAQFTINSYTATFDVDGETTNQKVDYQA
LLQEPTAPTKDGYTFVGWYDAKTGGTEWDFATSKMPTSDITLYARFTKN
PSSDNSQTAPGKDDKNDKDKLTIKANDSADATSTKLPKTSDDSSMIPTILGTLFIGGAILILRKKTTNI*
```

Region A / Region B

InlH (548 amino acids)

```
                                                       ↓
MKKRWNSVFKLVLMVTAILGLSLYVTTSQGVEVRAESITQPTAINVIFP
DPALANAIKIAAGKSNVTDTVTQADLDGITTLSAFGTGVTTIEGVQYLN
NLIGLELKDNQITDLTPLKNLT
KITELELSGNPLKNVSAIAGLQ
SIKTLDLTSTQITDVTPLAGLS
NLQVLYLDINQITNISPLAGLT
NLQYLSIGNAQVSDLTPLANLS
KLTTLKADDNKISDISPLASLP
NLIEVHLKNNQISDVSPLANTS
NLFIVTLTNQTITNQPVFYQNN
LVVPNVVKGPSGAPIAPATISDNGTYASPNLTWNLTSFINNVSYT
FNQSVTFKNTTVPFSGTVTQPLTEAYTAVFDVDGKQTSVTVGANE
LIKEPTAPTKEGYTFTGWYDAKTGGTKWDFATDKMPAEDITLYAQFTINSYTATFDIDGKLTTQKVTYQS
LLEEPVAPTKDGYTFTGWYDAKTGGTKWDFATGKMPAGNITLYAQFTKN
DNPNPDDPTTNTPTGNGDGTSNPSNSGGNTTLPTAGDENTMLPIFIGVFLLGTATLILRKTIKVK*
```

Region A / Region B

InlE (499 amino acids)

```
                                                     ↓
MKRNKTALRILVTLAVVLAITFWVGMSSKEVQAAVIEHPTPINEIFT
DPVLTDNVKTLLGKADVTDEVTQTDLDSVTHLSAKSAGITTIEGLQYLT
NLSELELIDNQVTDLNPLTNLT
KITELRLSGNPLKDVSALAGLK
NLKTMDLIYTDITDVTPLAGLS
NLQVLNLDINQITDITPLAGLS
NLQFLSFGSTQVSDLTPLANLS
KLTTLNAMNSKVSDVSPLTGLS
NLTEVYLEENQISDVSPLAKLP
NLSIVTLTNQTITNQPVFYQNK
PIVPNVVTGLSGELIAPDTISDNGTYTSPNLTWDLNSFINSVSYT
FNQSVTFKNTTAPFSGTVTQPLTEVYAVVFDVDGEQTSAMVGVNE
LINEPTAPAKEGYIFDGWYDAKTDGNKWDFGIDKMPASDITLYAKFT
ENEEPNASSPINVEPNDNNS
DNAEPNASSSNNVQENGTNE
GINNLNSSGEDKVNIKLPITGDKLNVLPIFVGAVLIGIGLVLFRKKRQTK*
```

Region A / Reg. B / Region D

Figure 2.4: Amino acid sequences of the new internalins InlG, InlH and InlE. Signal sequences are underlined. Putative signal peptidase 1 cleavage sites are marked by vertical arrows. Regions A, B and D are indicated. Amino acids of the leucine-rich repeats corresponding to the consensus sequence - L - - L - L - - N - L - D I - - L - - L - and conserved amino acids of the regions B and D (compared to the region B of InlA) are indicated by grey boxes. The cell wall anchor motifs LPKTS, LPTAG and LPITG are boxed.

2 and 3 of InlA, respectively. At the C-terminus, InlG possesses a putative cell wall anchor similar to that of InlA with the motif LPKTS, followed by a region of hydrophobic amino acids and a tail of positively charged residues. Surprisingly, this motif differs from the consensus sequence LPXTG by the exchange of glycine by serine. The deduced InlG protein shows 50% identity (57% similarity) to InlA. However, InlG is much smaller than InlA (490 amino acids compared to 800). This difference in size is mostly due to the lack in InlG of 10 LRR units in region A and one repeat of 70 amino acids in region B compared to the InlA protein. A computer-based sequence alignment of both proteins revealed that the LRRs of InlG correspond to the InlA repeats number 1, 4, 5+9 and 10, whereas 5+9 represents a LRR unit generated by the fusion of InlA repeats number 5 and 9 with the concomitant deletion of the repeats 6, 7 and 8 (App. C). This finding suggests that different internalins may arise by varying the number of LRRs and B repeats, whereas the inter-repeat region as well as the N- and C-terminal part of the proteins are basically conserved.

The *inlH* gene codes for a protein of 548 amino acids with seven LRR units in region A and, like InlG, two repeats of 70 and 49 amino acids in region B. The A repeats of InlH are similar to the LRRs number 1, 2, 3, 4, 5+9, 10 and 11 of InlA (App. C). The putative cell wall anchor of this protein contains the motif LPTAG. Although the deduced InlH protein has the same length as the previously described InlC2 (Dramsi *et al.*, 1997), these proteins are not identical. As shown in Fig. 2.2A, the amino acid sequences of InlC2 and InlH are the same in the first two-thirds of the molecules (except for one amino acid), but differ considerably at the C-terminus. Surprisingly, in this region the sequence of InlH is identical to that of InlD (also except for one amino acid), although the overall identity between the entire proteins is only 65%. It is worthwhile noting that all three proteins, InlC2, InlD and InlH, display the same cell wall anchor motif LPTAG, in which threonine is exchanged by alanine compared to the consensus pentapeptide LPXTG.

The *inlE* gene characterized in this study revealed 99.6% identity to the previously described *inlE* gene (Dramsi *et al.*, 1997). Sequence alignment shows that the two proteins are identical except for three amino acids (exchange of K by T, I by N and E by K at positions 115, 440 and 472, respectively, relative to InlE of the *inlC2DE* gene cluster). As shown in Fig. 2.4, the deduced InlE protein is composed of 499 amino acids. Like InlH, InlE contains seven LRR units in region A which display the same similarity to the LRRs of InlA as that shown by the repeats of InlH (App. C). InlE is anchored to the cell wall by the pentapeptide LPITG, which perfectly fits the consensus motif LPXTG. Interestingly, compared to the other internalins InlE possesses a smaller region B with only one repeat of 47 amino acids and additionally a third region of two repeats composed of 20 amino acids each, designated as region D. Computer-based amino acid sequence alignments revealed that region D shows similarity to the B repeats and might be therefore generated by deletion events within these homologous repeats.

2.4   The internalin family consists of highly conserved proteins

The structure of the internalins is highly conserved. As shown in Fig. 2.5, all large members of this protein family are composed of similar structural elements: a signal sequence, a leucine-rich repeat region (LRR or A region) consisting of 4 to 14 LRR units, a conserved inter-repeat region, a region B containing 1 to 4 repeats, and a cell wall anchor with the pentapeptide LPXTG as consensus sequence. InlB is the only large internalin which lacks this anchor sequence and is linked to the bacterial surface via the GW modules of the C repeats (Braun *et al*., 1997).
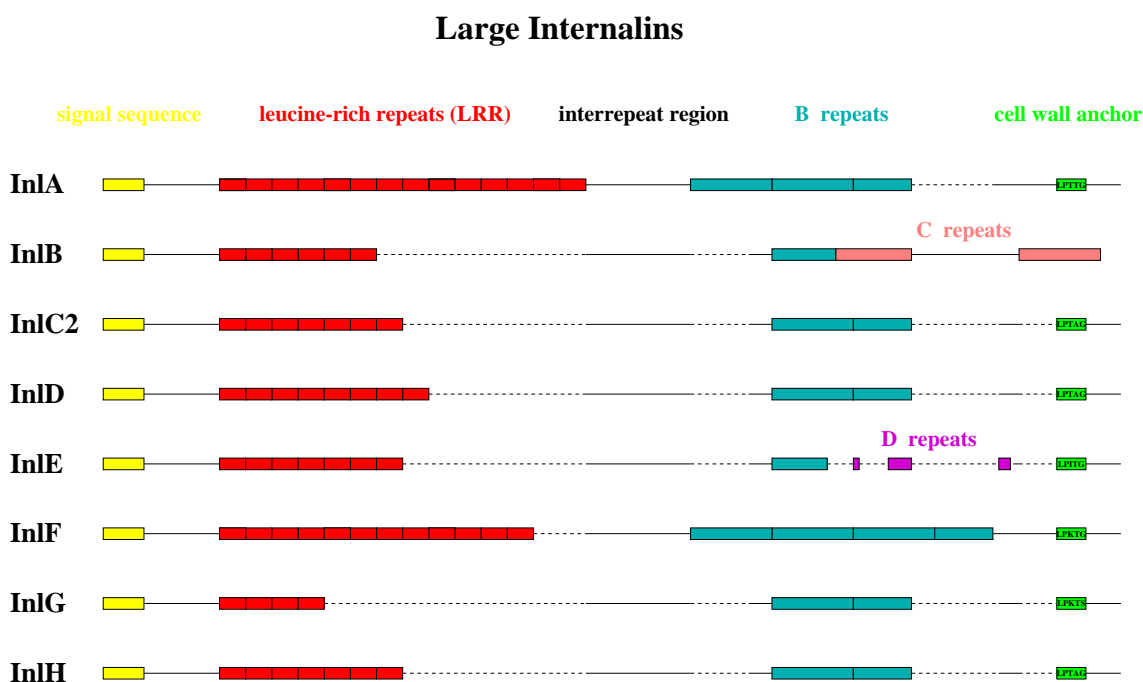
Figure 2.5: Comparison of the structure of the large internalins of *L. monocytogenes* EGD based on multiple alignment of the amino acid sequences generated by the program pileup (HUSAR, German Cancer Research Center). The structural elements signal sequence, leucine-rich repeats (LRR), inter-repeat region, B repeats, C repeats (of InlB), D repeats (of InlE) and cell wall anchor are indicated. LRR units were determined applying the definition adapted from (Kobe and Deisenhofer, 1994) and displayed consecutively according to their number. All other structures are organized based on amino acid sequence similarities. Dashed lines indicate inserted gaps.

A pairwise comparison of the amino acid sequence of the internalins performed with the program BestFit provided in the Genetics Computer Group software package is shown in Table 2.1. This study revealed that the proteins InlC2, InlD, InlE, InlG and InlH show the most pronounced homology among the internalins. The percent similarities vary from 98% between InlC2 and InlH to 58% between InlG and InlD. Among the proteins derived from the *inlGHE* gene cluster, similarity was highest between InlH and InlE (68%).This

striking conservation was also observed previously at the DNA level (Raffelsbauer, 1997). The high identity between the genes of the *inlGHE* cluster, especially between *inlH* and *inlE*, suggests that this cluster may have arisen by gene duplication. High similarity rates were also detected between the proteins InlC2, InlD and InlE (Dramsi *et al.*, 1997). In general, the proteins InlC2 to InlH seem to be more related to InlA than to InlB. Interestingly, InlB is the most divergent member of the internalin family and the small internalin InlC is more similar to InlB than to InlA. Fig. 2.6 shows an amino acid sequence alignment of the proteins InlG, InlH and InlE. In addition, a sequence alignment of all large internalins of *L. monocytogenes* EGD is included in App. C.

Table 2.1: Comparison of the amino acid sequences of internalins of *L. monocytogenes* EGD.

Inl protein (size)	Similarity of amino acid sequences in percent[a]						
	InlG	InlH	InlE[b]	InlC2	InlD	InlA	InlB
InlH (548 aa)	63 (555)						
InlE[b] (499 aa)	59 (417)	68 (549)					
InlC2 (548 aa)	59 (411)	98 (548)	74 (411)				
InlD (567 aa)	58 (416)	72 (571)	62 (436)	75 (572)			
InlA (800 aa)	57 (378)	59 (426)	50 (348)	71 (800)	69 (799)		
InlB (630 aa)	47 (322)	49 (421)	50 (408)	53 (611)	55 (637)	60 (835)	
InlC (297 aa)	47 (294)	49 (331)	49 (340)	59 (343)	61 (369)	64 (493)	64 (318)

[a]Similarities were calculated based on alignments of the amino acid sequences performed with the program BestFit provided in the software package of the Genetics Computer Group of the University of Wisconsin. The length in amino acids of the sequences aligned is given in parentheses. Gaps inserted to maximize alignments were considered in the length. Comparisons of Inl proteins not involving InlG, InlH or InlE were obtained from Dramsi *et al.* (1997).

[b]The sequence of the InlE protein as published in Raffelsbauer *et al.* (1998) was used for comparison.

2.5 Transcriptional studies of the genes *inlG*, *inlH* and *inlE* using a GFP-based expression system

The previous report (Dramsi *et al.*, 1997) showed a very weak expression of the three genes, *inlC2*, *inlD* and *inlE*. Therefore, a more sensitive GFP-based expression system described in Bubert *et al.* (1999) was used to test for transcription of the genes *inlG*, *inlH* and *inlE*. As depicted in Fig. 2.7, a *Listeria-E. coli* shuttle vector was constructed by fusing the vectors pUC18 and pBCE-16. The created plasmid pLSV16*gfp* (Bubert *et al.*, 1999) carries the cDNA *gfp*-mut2 encoding the green fluorescent protein GFP (Cormack *et al.*, 1996).

In the present study, the expected regulatory regions upstream of the three genes *inlG*, *inlH* and *inlE* were amplified by PCR using primers which carry *Xba*I restriction sites. These PCR products were cloned into the reporter plasmid pLSV16*gfp* via *Xba*I, yielding the plasmids P*inlG-gfp*, P*inlH-gfp* and P*inlE-gfp*, respectively (Fig. 2.7). The putative promoter regions of the *inl* genes were linked to the promoterless *gfp* cDNA flanked by a

```
InlE ~MKRNKTALR ILVTLAVVLA ITFWVGMS.S KEVQAAVIEH PTPINEIFTD PVLTDNVKTL  58
InlH MKKRWNSVFK LVLMVTAILG LSLYVTTSQG VEVRAESITQ PTAINVIFPD PALANAIKIA  60
InlG MKQRKTSVLH VLLVVTAILG ISLWVNASHG MKAQAESIAQ PAPINEIFTD PALADEVKTE  60

InlE LGKADVTDEV TQTDLDSVTH LSAKSAGITT IEGLQYLTNL SELELIDNQV TDLNPLKNLT 118
InlH AGKSNVTDTV TQADLDGITT LSAFGTGVTT IEGVQYLNNL IGLELKDNQI TDLTPLKNLT 120
InlG LGKTSVTDEV TQTDLNQITK LEADDKGINS IEGIQYLTNL NMLGVSSNQI TNITPLANLT 120

InlE KITELRLSGN PLKDVSALAG LKNLKTMDLI YTDITDVTPL AGLSNLQVLN LDINQITDIT 178
InlH KITELELSGN PLKNVSAIAG LQSIKTLDLT STQITDVTPL AGLSNLQVLY LDLNQITNIS 180
InlG .......... .......... .......... .......... ....NLDSLY LGDNKISDVT 136

InlE PLAGLSNLQF LSFGSTQVSD LTPLANLSKL TTLNAMNSKV SDVSPLTGLS NLTEVYLEEN 238
InlH PLAGLTNLQY LSIGNAQVSD LTPLANLSKL TTLKADDNKI SDISPLASLP NLIEVHLKNN 240
InlG PLSGLTQLTF VQLSINQIKD VTPLANLTKL NYLD...... .......... ......LREN 174

InlE QISDVSPLAK LPNLSIVTLT NQTITNQPVF YQNKPIVPNV VTGLSGELIA PDTISDNGTY 298
InlH QISDVSPLAN TSNLFIVTLT NQTITNQPVF YQNNLVVPNV VKGPSGAPIA PATISDNGTY 300
InlG QISDASPLVN MTDLTVLHLE KQQITAAPVV YQTNLVAPDI LKNAYGEVVP PTTISNNGTF 234

InlE TSPNLTWDLN SFINSVSYTF NQSVTF.KNT TAPFSGTVTQ PLTEV...YA VVFDVDGEQT 354
InlH ASPNLTWNLT SFINNVSYTF NQSVTF.KNT TVPFSGTVTQ PLTEA...YT AVFDVDGKQT 356
InlG ASPNITWNLD SFTSEVSYDF NQKITLGDNG KVTFAGTVVQ PIVEAPVNYI TTFDVDGTTT 294

InlE SAMVGVNELI NEPTAPAKEG YIFDGWYDAK TDGNKWDFGI DKMPASDITL YAKFTEN... 411
InlH SVTVGANELI KEPTAPTKEG YTFTGWYDAK TGGTKWDFAT DKMPAEDITL YAQFTINSYT 416
InlG TENVVVDTLI TEPAEPTKEG YTFSGWYDAE TGGNEWDFAV DKMPATNMTL YAQFTINSYT 354

InlE .......... .......... EEPNASS... .......... .......... ...PI.NVEP 424
InlH ATFDIDGKLT TQKVTYQSLL EEPVAPTKDG YTFTGWYDAK TGGTKWDFAT GKMPAGNITL 476
InlG ATFDVDGETT NQKVDYQALL QEPTAPTKDG YTFVGWYDAK TGGTEWDFAT SKMPTSDITL 414

InlE NDNNSDN.AE PNASSSINVQ ENGTNEGINN LNSSGEDKVN IKLPITGDEL NVLPIFVGAV 483
InlH YAQFTKN.DN PNPDDPTTNT PTGNGDGTSN PSNSGG...N TTLPTAGDEN TMLPIFIGVF 532
InlG YARFTKNPSS DNSQTAPGKD DKNDKDKLTI KANDSADATS TKLPKTSDDS SMIPTILGTL 474

InlE LIGIGLVLFR KKRQTK*499
InlH LLGTATLILR KTIKVK*548
InlG FIGGAILILR KKTTNI*490
```

Figure 2.6: Multiple alignment of the amino acid sequences of the proteins InlG, InlH and InlE generated using the programs pileup and prettybox of the HUSAR resource from the German Cancer Research Center. Identical and similar amino acids are indicated by black and grey boxes, respectively. Numbers at the right margin indicate the position of the corresponding amino acids. Inserted gaps are represented by dots.

ribosome binding site (AGGAG). Correct cloning was confirmed by sequence analysis of the plasmids. GFP-mediated fluorescence could be seen macroscopically in *E. coli* due to the high copy number of the plasmids in this strain. In *L. monocytogenes*, where the copy number of the plasmids is approximately 30 per bacterium, no fluorescence could be visualized macroscopically.

The plasmids P*inlG-gfp*, P*inlH-gfp* and P*inlE-gfp* were introduced either into the strain *L. monocytogenes* wild type or into an isogenic Δ*prfA* mutant (Böckmann *et al.*, 1996) by electroporation. In addition, wild type strains carrying the GFP plasmids were complemented with additional copies of *prfA* encoded on the plasmid pERL3502 (Leimeister-Wächter *et al.*, 1990). Clones were selected by tetracycline (for pLSV16*gfp* derived plasmids) or erythromycin (for pERL3502) resistance and screened by PCR. As shown in Fig. 2.8, PCR screening using appropriate primers yielded DNA fragments of the expected sizes, demonstrating that the selected clones harbored the corresponding plasmids.

The promoter activity of each of the genes *inlG*, *inlH* and *inlE* of *L. monocytogenes* grown in BHI to the logarithmic phase was determined by measuring the GFP-mediated fluorescence as described in detail in section 5.3.1. The GFP plasmids containing the promoters of the *L. monocytogenes* genes *inlA*, *actA* and *hly* described in Bubert *et al.* (1999) were used in this study for comparison. As shown in Fig. 2.9A the DNA fragment containing the regulatory region of *inlG* yielded the highest GFP expression, while the two other regulatory sites, the intergenic regions between *inlG* and *inlH* and between *inlH* and *inlE*, resulted in a significantly lower expression. In front of all three *inl* genes, and especially upstream of *inlG*, there are several putative promoter sequences which could serve as start sites for transcription of each of these genes. In addition, the DNA sequences of the intergenic regions between *inlG* and *inlH*, and between *inlH* and *inlE* also contain putative transcriptional terminators (see Fig. 2.1), suggesting the monocistronic nature of the three *inl* gene transcripts.

Several virulence genes of *L. monocytogenes* including *inlA*, *inlB* and *inlC* are regulated by the transcriptional activator protein PrfA (Bohne *et al.*, 1996; Engelbrecht *et al.*, 1996). A search for the 14-bp dyad-symmetric sequence termed PrfA-box, which is recognized by this regulator, failed to detect the conserved target sequence TTAACANNT-GTTAA. Accordingly, GFP expression in none of the three constructs P*inlG-gfp*, P*inlH-gfp* and P*inlE-gfp* was affected in the *prfA*-negative mutant or in the strain complemented with additional copies of *prfA*. These data suggest that transcription of the genes *inlG*, *inlH* and *inlE* is not dependent on PrfA. To further exclude a putative activation of these genes by PrfA, a MEM shift experiment was performed. This shift from the rich medium BHI into MEM (minimum essential medium) leads to the transcriptional activation of most PrfA-regulated genes (Bohne *et al.*, 1994; Bohne *et al.*, 1996). Again no activation of the *inl* genes could be detected (Fig. 2.9A), confirming the independence of the new genes *inlG*, *inlH* and *inlE* on PrfA. Interestingly, transcription of *inlE* was reduced in the Δ*prfA* mutant, but not increased in the *prfA* complemented strain nor after the MEM shift.

To examine expression of the genes *inlG*, *inlH* and *inlE* under intracellular conditions, the wild type strains harboring the GFP constructs were used in cellular invasion assays with the epithelial cell line Caco-2. As shown in Fig. 2.9B none of the constructs yielded a significant expression of GFP, suggesting that transcription of the three *inl* genes
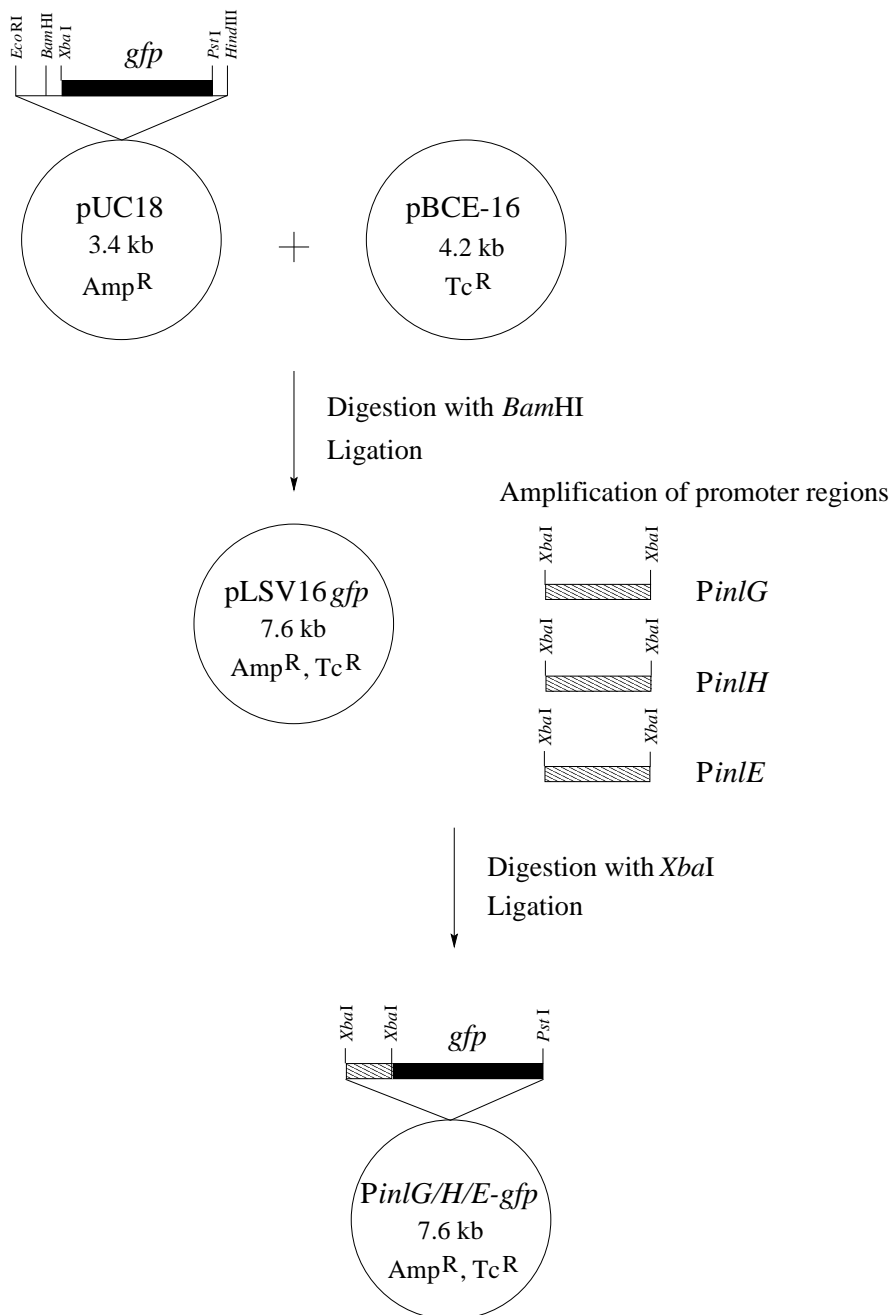
Figure 2.7: Construction of the GFP expression plasmids P*inlG-gfp*, P*inlH-gfp* and P*inlE-gfp*. The cDNA *gfp*-mut2 (Cormack *et al*., 1996) encoding the green fluorescent protein was cloned into the vector pUC18, which was then fused to the vector pBCE-16, yielding the shuttle vector pLSV16*gfp* (Bubert *et al*., 1999). The promoter regions of the genes *inlG*, *inlH* and *inlE* were amplified by PCR and cloned into pLSV16*gfp* via the *Xba*I restriction site linked to the promoterless *gfp* cDNA flanked by a ribosome binding site, yielding the plasmids P*inlG-gfp*, P*inlH-gfp* and P*inlE-gfp*, respectively.

A

B

C1

C2

C3
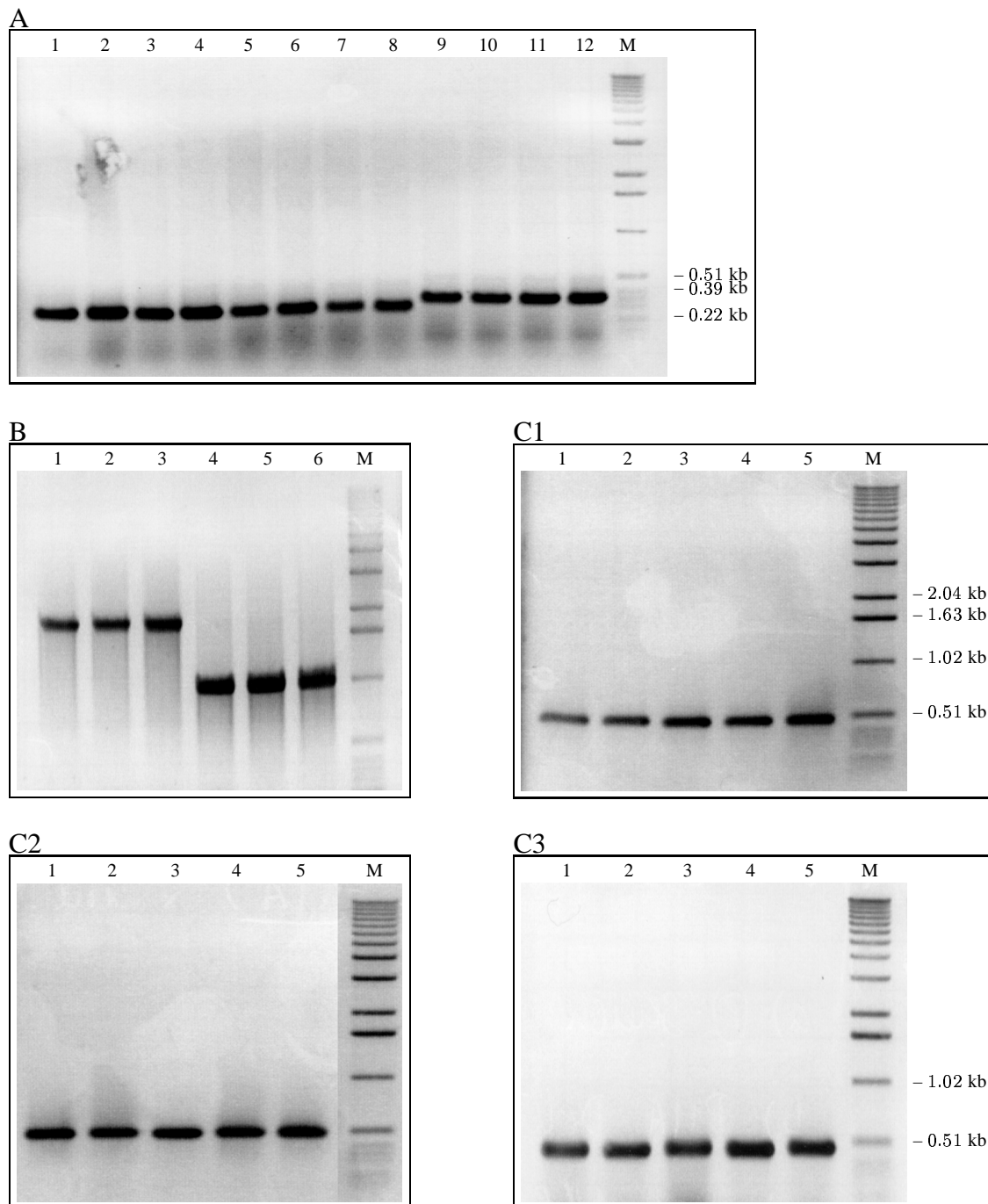
Figure 2.8: PCR analysis of *L. monocytogenes* clones carrying GFP expression plasmids. A. PCR screening to detect the plasmids P*inlG-gfp* (lanes 1-4), P*inlH-gfp* (5-8) and P*inlE-gfp* (9-12) in the wild type strain. B. PCR screening to detect clones with wild type *prfA* (lanes 1-3) and mutant *prfA* (lanes 4-6) carrying the plasmids P*inlG-gfp* (1 and 4), P*inlH-gfp* (2 and 5) and P*inlE-gfp* (3 and 6). C. PCR screening of strains complemented with additional copies of *prfA* carried on the plasmid pERL3502, harboring in addition the plasmids P*inlG-gfp* (C1), P*inlH-gfp* (C2) and P*inlE-gfp* (C3).

is not activated during intracellular replication in these cells, in contrast to other known PrfA-regulated virulence genes (Bubert *et al.*, 1999). Experiments using GFP-based expression plasmids were performed together with Dr. A. Bubert and Dr. A. Simm from the University of Würzburg, Germany. The corresponding data, which were published in Raffelsbauer *et al.* (1998), are cited here with their kind permission.

2.6   Construction of an in-frame *inlGHE* deletion mutant

The previous study did not reveal any effect of *inlC2*, *inlD* and *inlE* on the virulence of *L. monocytogenes* (Dramsi *et al.*, 1997). In order to assess a putative contribution of the newly identified genes *inlG* and *inlH* to the bacterial virulence, an in-frame deletion mutant which had lost the genes *inlG*, *inlH* and *inlE* was constructed as described in Raffelsbauer (1997). A schematic representation of the strategy used to construct this mutant is shown in Fig. 2.10. In brief, deletion was achieved by double cross-over using a pLSV1-based knock-out vector termed pLSVΔ*inlGHE*. To construct this plasmid, short DNA fragments (GA and EB) were amplified from the 5' regions of the *inlG* and *dapE* genes, respectively, ligated via a *Psp*A1 restriction site and cloned into the vector pLSV1, which carried in addition an erythromycin resistance gene (Em^{R}) and a temperature-sensitive Gram-positive origin of replication (Ori$_{ts}$). Integration of the knock-out vector in the chromosome was induced by incubating bacteria at 42°C in the presence of erythromycin. Homologous recombination at the 5' end (between fragments GA and GA') was selected by PCR screening with appropriate primers. A second cross-over event between fragments EB and EB' led to the excision of the genes *inlG*, *inlH* and *inlE* from the chromosome, together with the intervening vector sequence. By this deletion the first 56 bp of the coding region of *inlG* were fused in-frame to the last 34 bp of *inlE*, yielding a mutant *inlGE* fusion gene which is preceded by the *inlG* promoter and encodes a 29-amino acids long residual peptide. The correct in-frame deletion was confirmed by PCR and nucleotide sequence analysis of the shortened PCR product, as shown in App. B.2. Further details on the construction of the Δ*inlGHE* mutant are given in section 5.2.1.

2.7   Characterization of the *L. monocytogenes* Δ*inlGHE* mutant

To investigate a putative contribution of the *inlGHE* gene cluster to the virulence of *L. monocytogenes* the Δ*inlGHE* mutant was examined *in vivo* in the mouse model and *in vitro* using different cell lines.

2.7.1   Deletion of the *inlGHE* gene cluster reduces virulence

C57BL/6 mice were infected orally with 2×10^8 bacteria of the strains *L. monocytogenes* Δ*inlGHE* or wild type. The colony forming units in liver and spleen were then determined 1 and 5 days post-infection. As shown in Table 2.2 there is a reduction of approximately 3 and 2.5 $\log_{10}$ units in bacterial counts in liver and spleen, respectively, of the mutant
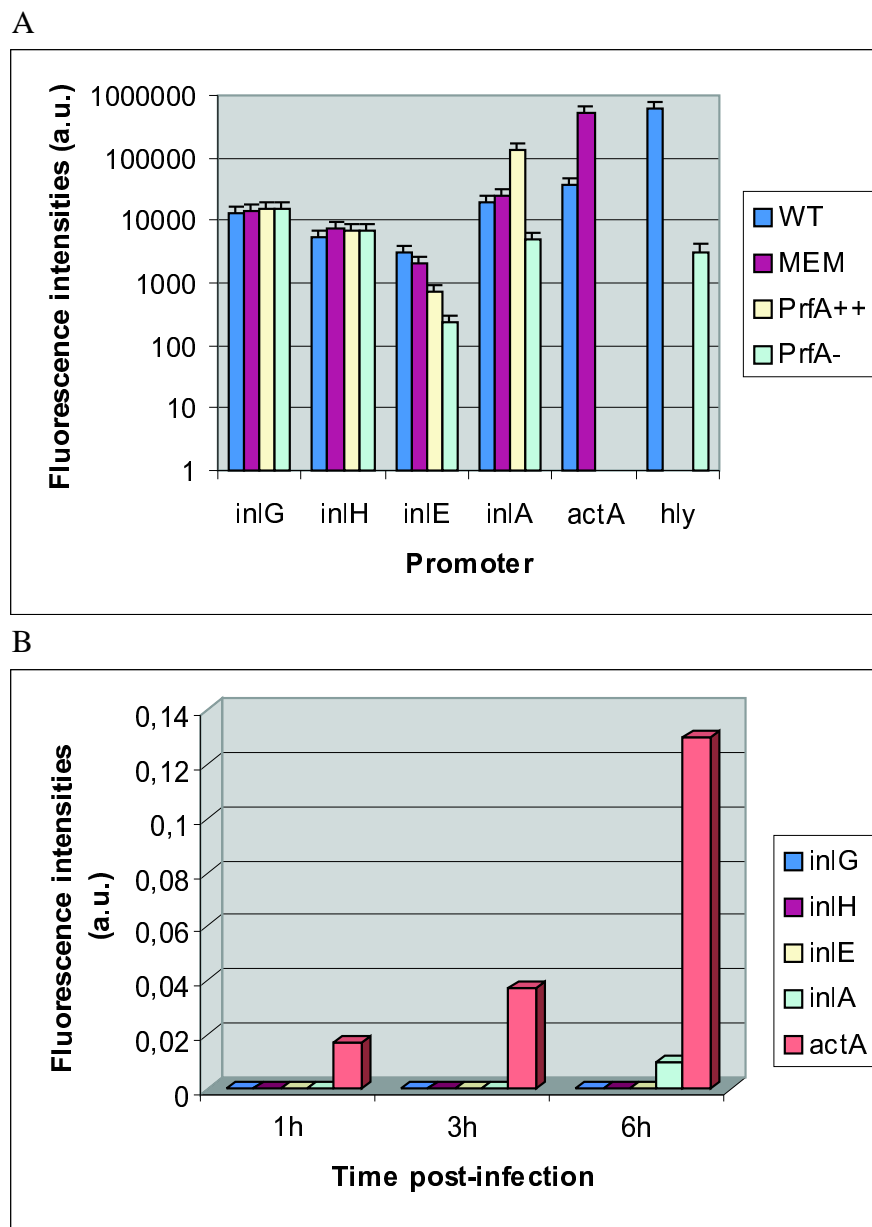
Figure 2.9: Fluorescence intensities in arbitrary units (a. u.) of *L. monocytogenes* wild type (WT), WT complemented with additional copies of *prfA* (PrfA++) and strain Δ*prfA* (PrfA−), all carrying the plasmid-encoded *gfp* cDNA under control of the *inlG*, *inlH*, *inlE*, *inlA*, *actA* and *hly* promoters, respectively. A. *L. monocytogenes* strains were grown in BHI to the logarithmic phase. Fluorescence was determined as described in detail in section 5.3.1. For the MEM shift experiments, bacteria were grown to the log phase in BHI and then shifted for 1 h into MEM. B. Fluorescence intensities of bacteria grown in Caco-2 cells. Cells were infected with *L. monocytogenes* strains transformed with the indicated GFP plasmids at a MOI of 50 bacteria per cell. The time points post-infection are indicated. Fluorescence levels of strains in measurements A and B cannot be directly compared because different numbers of bacteria and different blanks were used. Note that the P*actA* and P*hly* constructs were used as control for MEM shift experiments and Δ*prfA* strains, respectively, in addition to BHI-grown wild type strains.
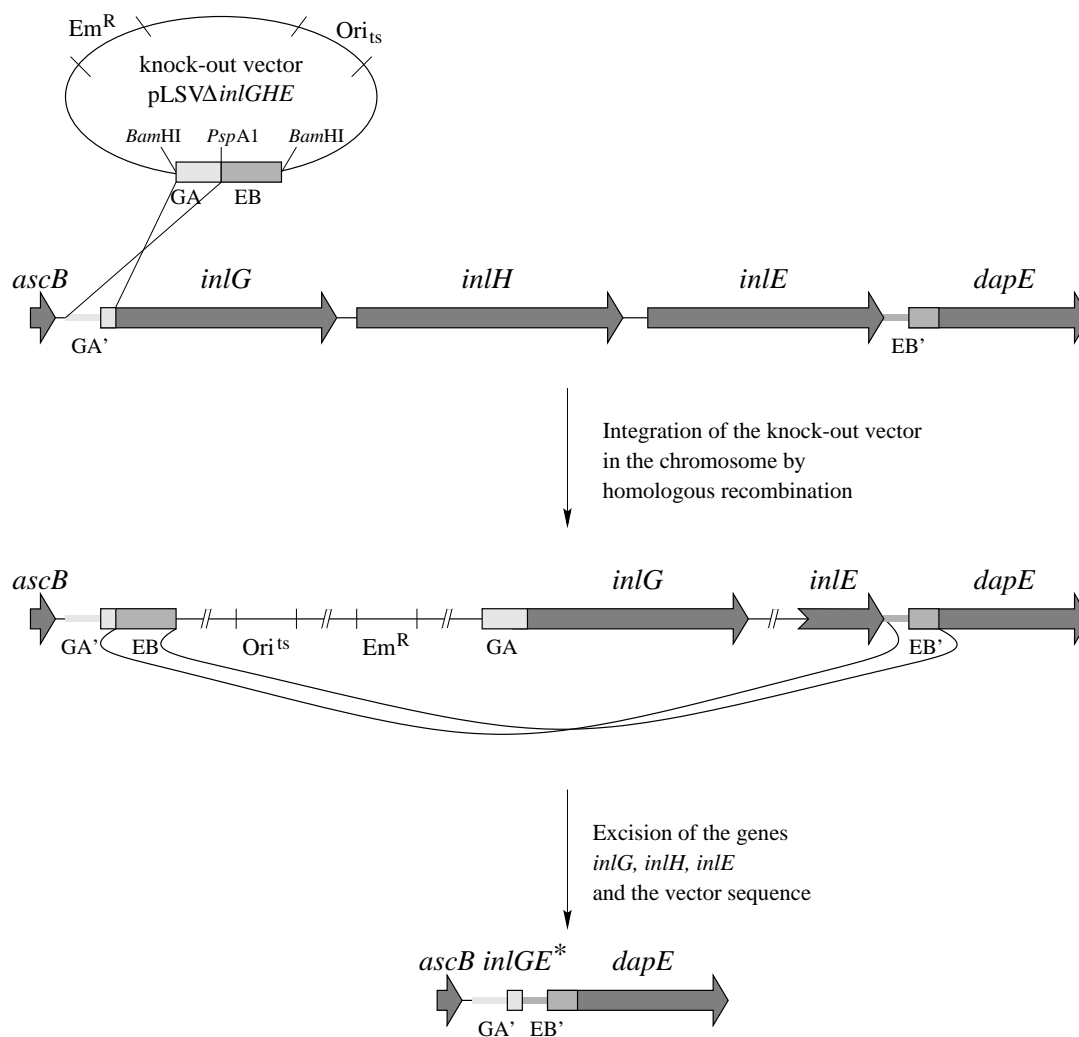
Figure 2.10: Strategy used for construction of the in-frame deletion mutant *L. monocyto-genes* Δ*inlGHE* by double cross-over. To construct the knock-out vector pLSVΔ*inlGHE* short fragments (GA and EB) were amplified from the 5' regions of the *inlG* and *dapE* genes, respectively, ligated via a *Psp*A1 restriction site and cloned into the vector pLSV1, which carried in addition an erythromycin resistance gene (Em^R) and a temperature-sensitive Gram-positive origin of replication (Ori$_{ts}$). Integration of the knock-out vector in the chromosome was induced by incubating bacteria at 42°C in the presence of erythromycin. Homologous recombination at the 5' end via fragments GA and GA' was selected by PCR screening with appropriate primers. Excision of the genes *inlG*, *inlH* and *inlE* from the chromosome, together with the intervening vector sequence was achieved by homologous recombination via fragments EB and EB' and confirmed by PCR. The resulting Δ*inlGHE* deletion mutant carries a truncated *inlGE* chimeric gene encoding a 29-amino acids residual peptide. The in-frame deletion was verified by nucleotide sequence analysis as shown in App. B.2.

compared to the wild type strain on day 1 after infection. This significant reduction of viable bacteria counts in liver and spleen suggests that the *inlGHE* deletion mutant is impaired in the proficient entry and/or replication in these two organs and argues for an important role of the *inlGHE* gene cluster in virulence. This experiment was performed by Dr. J. Hess and Prof. Dr. S. H. E. Kaufmann from the Max-Planck-Institute for Infection Biology, Berlin, Germany.

Table 2.2: Oral infection of mice with *L. monocytogenes* strains $\Delta inlGHE$ and wild type. Data cited with kind permission of Dr. J. Hess and Prof. Dr. S. H. E. Kaufmann (Raffelsbauer *et al.*, 1998).

Bacterial strain	Day p. i.	$\log_{10}$ CFU (mean $\pm$ SD) in[a]	
		livers	spleens
L. monocytogenes	1	1.92 ± 1.80	2.60 ± 1.70
$\Delta inlGHE$	5	2.24 ± 0.45	2.17 ± 0.41
L. monocytogenes	1	4.79 ± 1.01	5.02 ± 1.01
Wild type	5	3.75 ± 0.42	3.90 ± 0.35

[a]Determination of colony forming units (CFU) at day 1 and 5 p. i. in livers and spleens of C57BL/6 mice infected orally with 2×10^8 bacteria. The means $\pm$ SD per time point of five animals are presented. CFU results of both groups were statistically different on day 1 and day 5 as analyzed by unpaired t test ($p < 0.05$).

2.7.2 *In vitro* studies using the $\Delta inlGHE$ mutant

In order to examine the reduction of virulence observed with the *L. monocytogenes* EGD $\Delta inlGHE$ mutant in the mouse model in more detail and to determine whether the mutant is impaired in the ability to invade into or grow in mammalian cells, this strain was tested *in vitro* for uptake and replication in the human enterocyte-like cell line Caco-2, which is routinely used for the analysis of invasion, intracellular replication and intercellular spreading of *L. monocytogenes*. The intracellular growth of the bacteria was studied using the plasmid P*actA-gfp*, in which expression of GFP is under control of the *actA* promoter, a listerial promoter activated in the host cell cytosol (Dietrich *et al.*, 1998). As shown in Fig. 2.11, in the non-phagocytic cell line Caco-2 no significant difference in intracellular replication and cell-to-cell spreading between the wild type and the mutant strain was observed 18 h post-infection using the GFP expression vector P*actA-gfp*. This result indicates that the $\Delta inlGHE$ mutant replicates within Caco-2 cells as efficiently as the wild type strain. Note that the invasion rate of the bacteria was not determined in this assay.

Next, invasion and replication of the $\Delta inlGHE$ mutant were examined using the murine professional phagocytic cell line J774. In contrast to the observation obtained with the cell line Caco-2, in the macrophage-like J774 cells the number of *gfp* expressing mutant bacteria visualized 18 h post-infection was significantly lower than that of the wild type strain

A

B

C

D
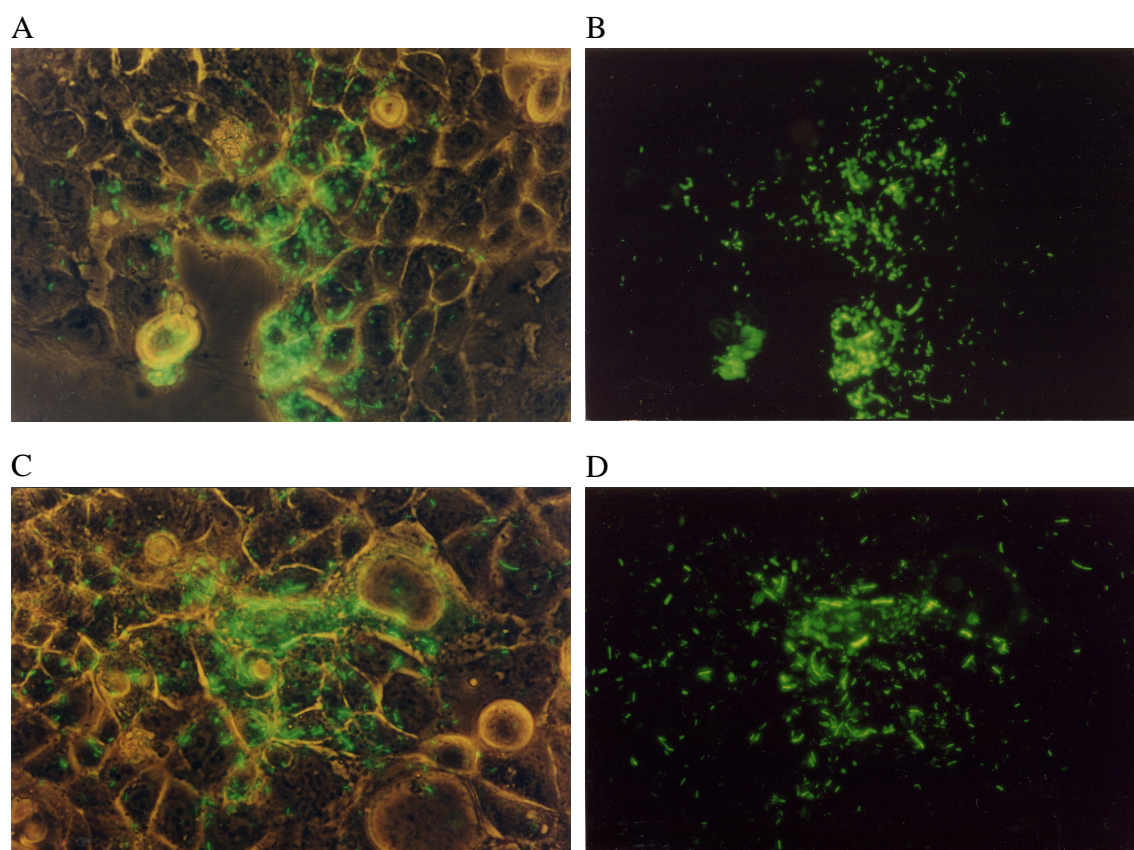
Figure 2.11: A–D. Invasion, intracellular replication and cell-to-cell spreading of *L. monocytogenes* wild type (A, B) and Δ*inlGHE* mutant (C, D) in Caco-2 cells. Both strains were previously transformed with the P*actA-gfp* plasmid and used in a MOI of 20 bacteria per cell for these microscopical studies. (A, C) combined phase contrast and fluorescence pictures, and (B, D) fluorescence pictures of the same regions of the monolayers documented 18 h post infection. Pictures shown with kind permission of Dr. A. Bubert (Raffelsbauer *et al*., 1998).

(data not shown). A FACS analysis of the intracellular replication of the ∆*inlGHE* mutant containing the plasmid P*actA-gfp* in these cells confirmed that the total fluorescence intensities of the mutant were lower than that of the parental strain at different time points post-infection (Fig. 2.12). This more detailed study also revealed that the number of infected cells was similar for both strains, suggesting that the mutant was taken up by the J774 cells as efficiently as the wild type. However, the fluorescence intensity emitted by the mutant in each of the infected cells was strongly reduced, resulting in the lower total fluorescence observed with this strain. The ability of the ∆*inlGHE* mutant to adhere to and invade into phagocytic cells of the cell lines J774 and P388 was then examined in adhesion and cellular invasion assays and was indeed similar to that of the wild type strain. Fig. 2.13 shows the ability of the ∆*inlGHE* mutant to invade P388 cells in comparison to wild type strain.
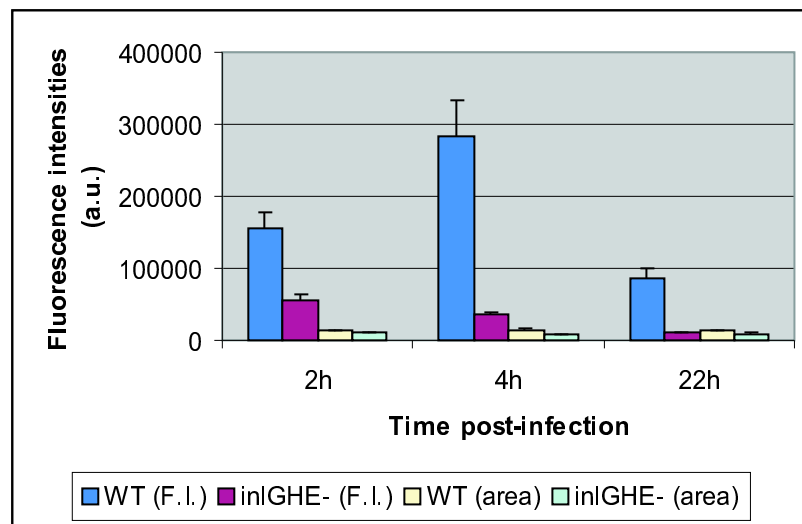
Figure 2.12: FACS analysis of J774 cells infected with *L. monocytogenes* wild type and ∆*inlGHE* mutant strains containing the GFP expression plasmid P*actA-gfp*. Graphs show the number of fluorescence positive, i. e. infected cells (area) and the total fluorescence intensity (F.I.), which is the product of the area and the mean of fluorescence per cell. 20,000 cells were analyzed per sample. Fluorescence intensities are given in arbitrary units (a. u.). Experiment was performed in triplicate. Bars represent standard deviations.

Internalization of *L. monocytogenes* into non-phagocytic cells depends on the internalins InlA and InlB (Gaillard *et al.*, 1991; Dramsi *et al.*, 1995). To examine whether the identified internalins InlG, InlH and InlE are involved in this entry process, the ∆*inlGHE* mutant was tested for internalization into the human enterocyte-like Caco-2 and the murine hepatocyte-like TIB73 cells. As shown in Fig. 2.13, the invasion rate of the mutant into these cell lines was two- to three-fold higher than that of the wild type strain. A similar increase of invasiveness in the absence of *inlGHE* was also observed with human brain microvascular endothelial cells (Greiffenberg, 2000). The above data thus suggest that InlG, InlH and InlE do not trigger uptake into non-phagocytic mammalian cells directly,

but seem to negatively interfere with InlA- and/or InlB-mediated bacterial internalization.
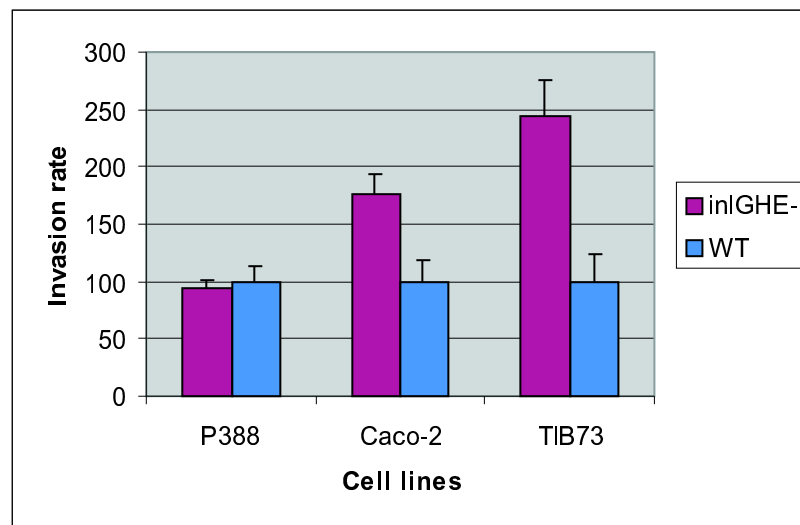
Figure 2.13: Uptake of the *L. monocytogenes* strains $\Delta inlGHE$ and wild type by different mammalian cells. Entry of the mutant strain is given as the rate of invasion relative to that of the wild type strain, which was set to 100. The cell lines used were P388 (murine macrophage-like), Caco-2 (human enterocyte-like) and TIB73 (murine hepatocyte-like). Experiments were performed in triplicate. Bars represent standard deviations.

2.8 Construction of new $\Delta inlGHE$ mutant strains

To rule out additional mutations on the chromosome of the $\Delta inlGHE$ mutant that could eventually affect bacterial uptake, three new $\Delta inlGHE$ mutant strains (termed S57, S58 and S59) were constructed using the same strategy applied for the construction of the first $\Delta inlGHE$ mutant (S14) shown in Fig. 2.10 and described in detail in section 5.2.1. By double cross-over using the knock-out plasmid pLSV$\Delta inlGHE$, most of the *inlGHE* gene cluster was removed. Integration of pLSV$\Delta inlGHE$ into the chromosome and deletion of the genes by homologous recombination were confirmed by PCR screening, which yielded products as depicted in Fig. 2.14. By this deletion the first 56 bp of the coding region of *inlG* were fused in frame to the last 34 bp of *inlE*, yielding a mutant *inlGE* fusion gene which is preceeded by the *inlG* promoter and encodes a 29-amino acids long residual peptide. The correct in-frame deletions were confirmed by DNA sequence analysis of the shortened PCR products. The nucleotide sequences of the constructed $\Delta inlGHE$ mutant strains are shown in App. B.2. When tested in cellular invasion assays, the new $\Delta inlGHE$ mutant strain S57 also showed increased invasion rates in non-phagocytic cells similar to those observed with the first $\Delta inlGHE$ mutant S14 (B. Bergmann, unpublished data). These results indicate that the increase of invasiveness is specifically caused by the deletion of the *inlGHE* gene cluster from the *L. monocytogenes* EGD chromosome.
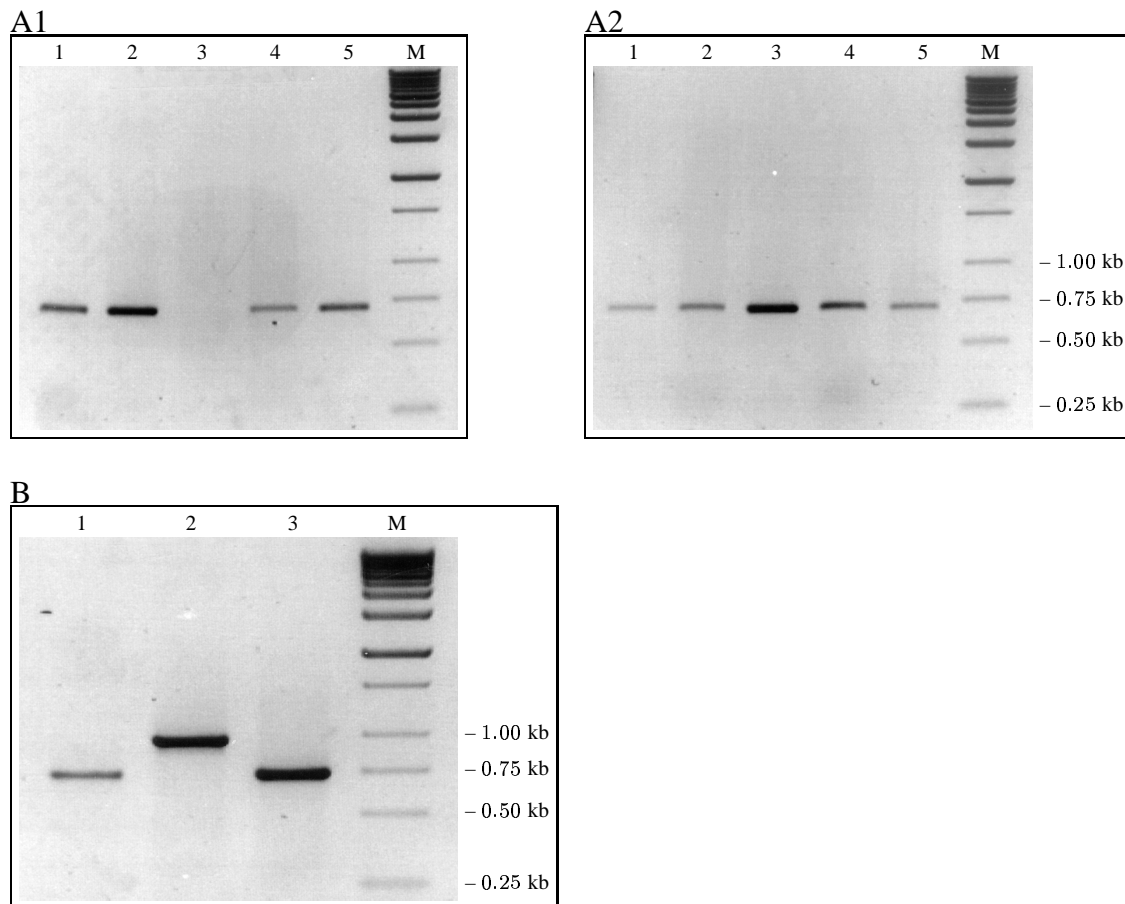
Figure 2.14: Construction of new *L. monocytogenes* Δ*inlGHE* mutant strains. PCR analysis to isolate single and double cross-over mutants. A. Integration of the knock-out plasmid pLSVΔ*inlGHE* into the chromosome was detected by PCR using the primers pGluco-1 and ORFZ-1, which hybridized to the *ascB* gene of the wild type sequence and to the homologous fragment EB of the knock-out plasmid, respectively. The size of the expected PCR product is 703 bp. Integration was performed in two independent experiments (A1 and A2). From each experiment, one single cross-over clone was used to construct a double cross-over mutant. B. Deletion of the *inlGHE* gene cluster was detected by PCR with the primers PinlXu and delxy-7, which hybridized to the *ascB* and *dapE* genes of the wild type sequence, respectively. Deletion of the *inlGHE* gene cluster yielded a PCR product of 728 bp instead of a 5,602-bp long DNA fragment of the wild type. Lanes 1, 2 and 3 correspond to the *L. monocytogenes* Δ*inlGHE* mutant strains S57, S58 and S59, respectively. PCR products shown in B were sequenced (App. B.2). M is the DNA molecular weight standard.

2.9 Construction and characterization of the *L. monocytogenes* single mutants Δ*inlG*, Δ*inlH* and Δ*inlE*

In order to test whether deletion of a single gene from the *inlGHE* gene cluster has a similar stimulatory effect on invasiveness of *L. monocytogenes* as the deletion of the complete gene cluster, single in-frame deletion mutants lacking either *inlG*, *inlH* or *inlE* were constructed in the present study. First, a Δ*inlG* mutant was constructed applying the same strategy used to construct the Δ*inlGHE* mutant using the pLSV1-based knock-out plasmid pLSVΔ*inlG*I. This mutant was constructed in duplicate and the obtained strains were termed Δ*inlG*I-1 (S38) and Δ*inlG*I-2 (S37). As depicted in Fig. 2.15, when tested in cellular invasion assays, both *inlG* deletion mutant strains showed a reduction in invasiveness into the two non-phagocytic cell lines Caco-2 and TIB73. The invasion rates of these mutants vary from 20% to 50% compared to that of the wild type strain. Nucleotide sequence analysis revealed that deletion of the *inlG* gene in these strains was not in frame. During the construction of plasmid pLSVΔ*inlG*I, digestion of fragments GA and GB with the enzyme *Psp*A1 caused the deletion of one nucleotide in the restriction site, leading to a frame shift. The resulting InlG peptide derived from the performed *inlG* deletion on the chromosome was 10 amino acids shorter than desired. To rule out that this shorter but much more hydrophobic peptide affects protein secretion and that the frame shift mutation alters the expression of the following genes, a second Δ*inlG* mutant, termed Δ*inlG*II, was constructed using a new knock-out plasmid pLSVΔ*inlG*II. In addition, the single mutants Δ*inlH* and Δ*inlE* were constructed.

A schematic representation of the strategy used to construct the single mutants Δ*inlG* (I and II) is shown in Fig. 2.16. The mutants Δ*inlH* and Δ*inlE* were constructed using the same method. In brief, deletion of the genes *inlG*, *inlH* and *inlE* was achieved by double cross-over using the pLSV1-based knock-out plasmids pLSVΔ*inlG* (I or II), pLSVΔ*inlH* and pLSVΔ*inlE* containing the homology fragments GAGB, HAHB and EAEB, respectively, which were used to delete the corresponding genes by homologous recombination. To construct each of these plasmids, short DNA fragments were amplified from the 5' and 3' regions of the gene to be deleted, ligated to each other via a *Psp*A1 restriction site, PCR amplified, digested and cloned into pLSV1 as described in detail in section 5.2.2. This shuttle vector carries an erythromycin resistance gene (Em^R) and a temperature-sensitive Gram-positive origin of replication (Ori$_{ts}$). Integration of the knock-out plasmids into the chromosome was induced by incubating bacteria at 42°C in the presence of erythromycin. Homologous recombination at the 5' end via fragments A and A' was detected by PCR screening with appropriate primers as shown in Fig. 2.17. The second cross-over via fragments B and B' leading to the excision of most of the wild type gene from the chromosome, together with the intervening vector sequence was accomplished by subculturing the bacteria in the absence of antibiotics. Deletion of the wild type genes was screened by PCR as depicted in Fig. 2.18, and confirmed by nucleotide sequence analysis of the obtained PCR products. The nucleotide sequences of the resulting single mutants are included in App. B.2. Deletions were in frame except for Δ*inlG*I. The mutants Δ*inlG*I, Δ*inlG*II, Δ*inlH* and Δ*inlE* carry truncated genes encoding 22-, 32-, 47- and 49-amino acids residual peptides, respectively. All single mutants were constructed
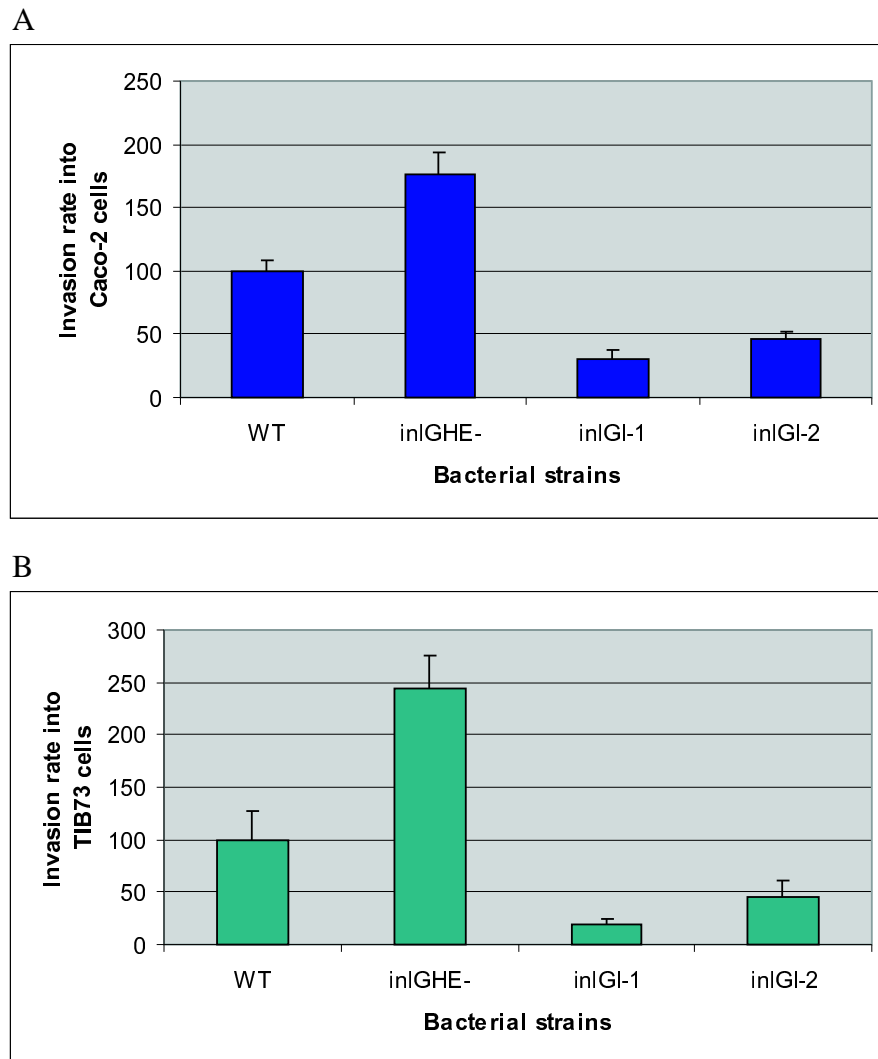
Figure 2.15: Internalization of *L. monocytogenes* strains wild type, Δ*inlGHE* and Δ*inlG*I into human enterocyte-like Caco-2 cells (A) and murine hepatocyte-like TIB73 cells (B). Cells were infected for 1 h at a MOI of 20 or 30 bacteria per Caco-2 or TIB73 cell, respectively. Invasiveness of the mutant strains is given as the rate of invasion relative to that of the wild type strain, which was set to 100. Experiments were performed in triplicate. Bars represent standard deviations.

in duplicate. Both selected clones of each single mutant were genetically characterized. These clones were designated as follows: $\Delta inlG$I: strains S37 and S38; $\Delta inlG$II: strains S53 and S54; $\Delta inlH$: strains S41 and S42; and $\Delta inlE$: strains S39 and S40. A schematic representation showing the genetic organization of the *inlGHE* gene cluster in the deletion mutants $\Delta inlG$, $\Delta inlH$, $\Delta inlE$ and $\Delta inlGHE$ compared to the wild type strain is depicted in Fig. 2.19.

The single deletion mutants $\Delta inlG$II, $\Delta inlH$ and $\Delta inlE$ were tested for internalization by HepG-2 cells. These experiments were performed by B. Bergmann from the University of Würzburg, Germany (Bergmann, Raffelsbauer *et al.*, manuscript submitted). These cellular invasion assays, which are cited here with the kind permission of B. Bergmann (ongoing doctoral thesis), revealed that, similarly to the $\Delta inlGHE$ mutant, each of the single mutants tested was also two- to three-fold more invasive than the wild type strain (Table 2.3). Thus, using the internalin mutants constructed in the present study it could be shown that either deletion of the complete *inlGHE* gene cluster or of the single genes *inlG*, *inlH* and *inlE* increases invasiveness of *L. monocytogenes* into non-phagocytic cells, suggesting that the internalins InlG, InlH and InlE may form a functional complex.

2.10 Construction and characterization of the *L. monocytogenes* revertant strains $inlG^+$, $inlH^+$ and $inlE^+$

In order to confirm that the increased invasiveness of the mutants defective in the genes *inlG*, *inlH* or *inlE* is caused by the desired deletions and not by additional mutations, the single mutants $\Delta inlG$ (I and II), $\Delta inlH$ and $\Delta inlE$ were restored by inserting a copy of the respective intact gene into the region of the previous chromosomal deletion by double cross-over, thereby reverting the mutants to the wild type genotype. Reversion was achieved using again the vector pLSV1 as shown in Fig. 2.20 and described in detail in section 5.2.2. In brief, the genes *inlG*, *inlH* and *inlE* were amplified from the chromosome of *L. monocytogenes* wild type using the primer pairs mutrevG-1/lisminv-1, delH-1/delH-4 and delE-1/delxy-6b, which were previously used to generate the homologous fragments GAGB, HAHB and EAEB applied to construct the deletion mutants $\Delta inlG$II, $\Delta inlH$ and $\Delta inlE$, respectively. The wild type genes were cloned into the vector pLSV1 via *Eco*RI (*inlG*) or *Bam*HI (*inlH* and *inlE*) restriction sites, yielding the knock-in plasmids pLSV*inlG*, pLSV*inlH* and pLSV*inlE*. Integration of these plasmids into the chromosome by homologous recombination at the 5' side, i. e. via fragments GA/GA', HA/HA' and EA/EA', was detected by PCR with appropriate primers as shown in Fig. 2.21. Restoration of the wild type gene after excision of the truncated gene and the vector sequence by homologous recombination via fragments GB/GB', HB/HB' and EB/EB' was screened by PCR using different primer combinations, which yielded products spanning the complete gene at once and additionally in smaller overlapping fragments, as depicted in Fig. 2.22 and 2.23. Appropriate PCR products were sequenced and the nucleotide sequences in the recombination regions were identical to those of the wild type strain (App. B.2). Bacterial strains used for reversion were S38 ($\Delta inlG$I), S54 ($\Delta inlG$II), S41 ($\Delta inlH$) and S40 ($\Delta inlE$). The truncated gene of both mutant strains $\Delta inlG$I and $\Delta inlG$II was replaced
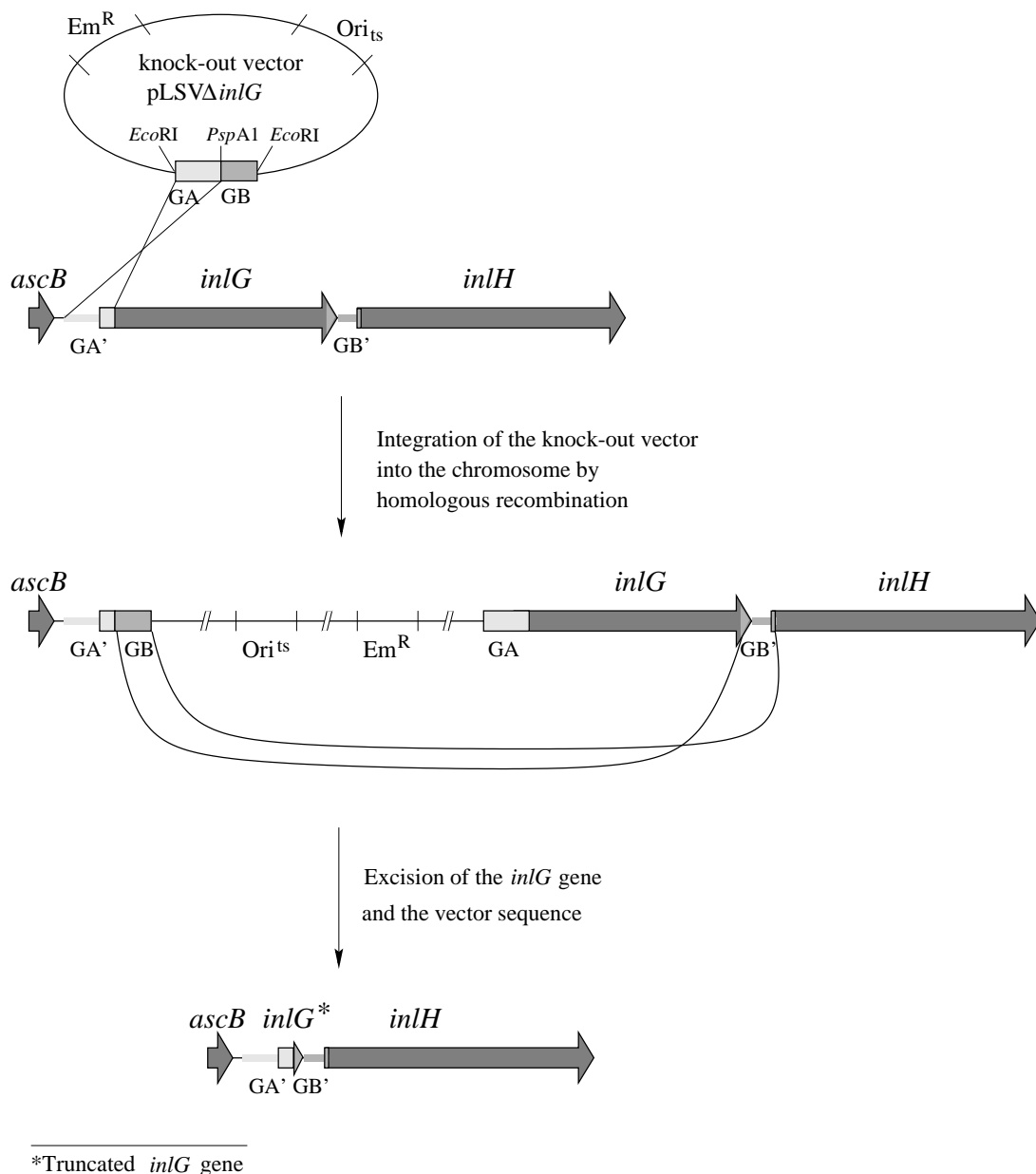
Figure 2.16: Strategy used for construction of the deletion mutants *L. monocytogenes* Δ*inlG*I and Δ*inlG*II by double cross-over. To construct the knock-out vector pLSVΔ*inlG* short fragments (GA and GB) were amplified from the 5' and 3' regions of the *inlG* gene, ligated via a *Psp*A1 restriction site and cloned into the vector pLSV1, which carried in addition an erythromycin resistance gene (Em^R) and a temperature-sensitive Gram-positive origin of replication (Ori$_{ts}$). Integration of the knock-out vector in the chromosome was induced by incubating the bacteria at 42°C in the presence of erythromycin. Homologous recombination at the 5' end (between fragments GA and GA') was detected by PCR screening with appropriate primers. A second cross-over event between fragments GB and GB' led to the excision of most of the *inlG* gene from the chromosome, together with the intervening vector sequence. The resulting Δ*inlG*I and Δ*inlG*II deletion mutants carry truncated *inlG* genes encoding 22- and 32-amino acids residual peptides, respectively. The deletions were confirmed by nucleotide sequence analysis as shown in App. B.2.
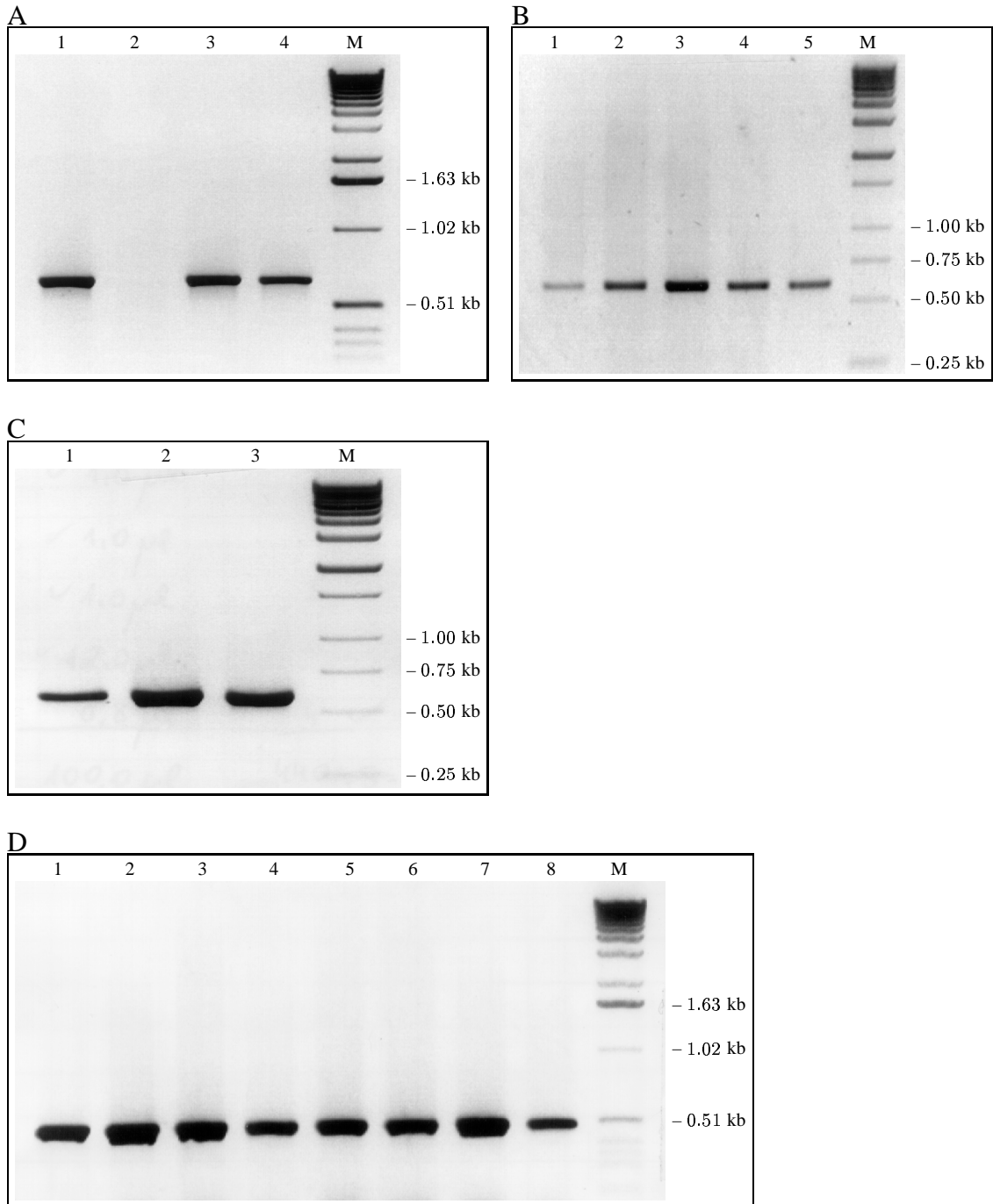
Figure 2.17: Construction of the *L. monocytogenes* single mutant strains ∆*inlG*I, ∆*inlG*II, ∆*inlH* and ∆*inlE*. PCR analysis to isolate single cross-over mutants. Integration of the knock-out plasmids pLSV∆*inlG*I (A), pLSV∆*inlG*II (B), pLSV∆*inlH* (C) and pLSV∆*inlE* (D) into the chromosome was detected by PCR using the primer pairs pGluco-1/PinlYd, pGluco-1/inlX-1, Xvorw-6/delHseq-2 and inlYseq-1/delxy-5, respectively. The sizes of the expected PCR products are 671 bp (∆*inlG*I), 599 bp (∆*inlG*II), 630 bp (∆*inlH*) and 490 bp (∆*inlE*). Two independent single cross-over clones were used to construct double cross-over mutants.
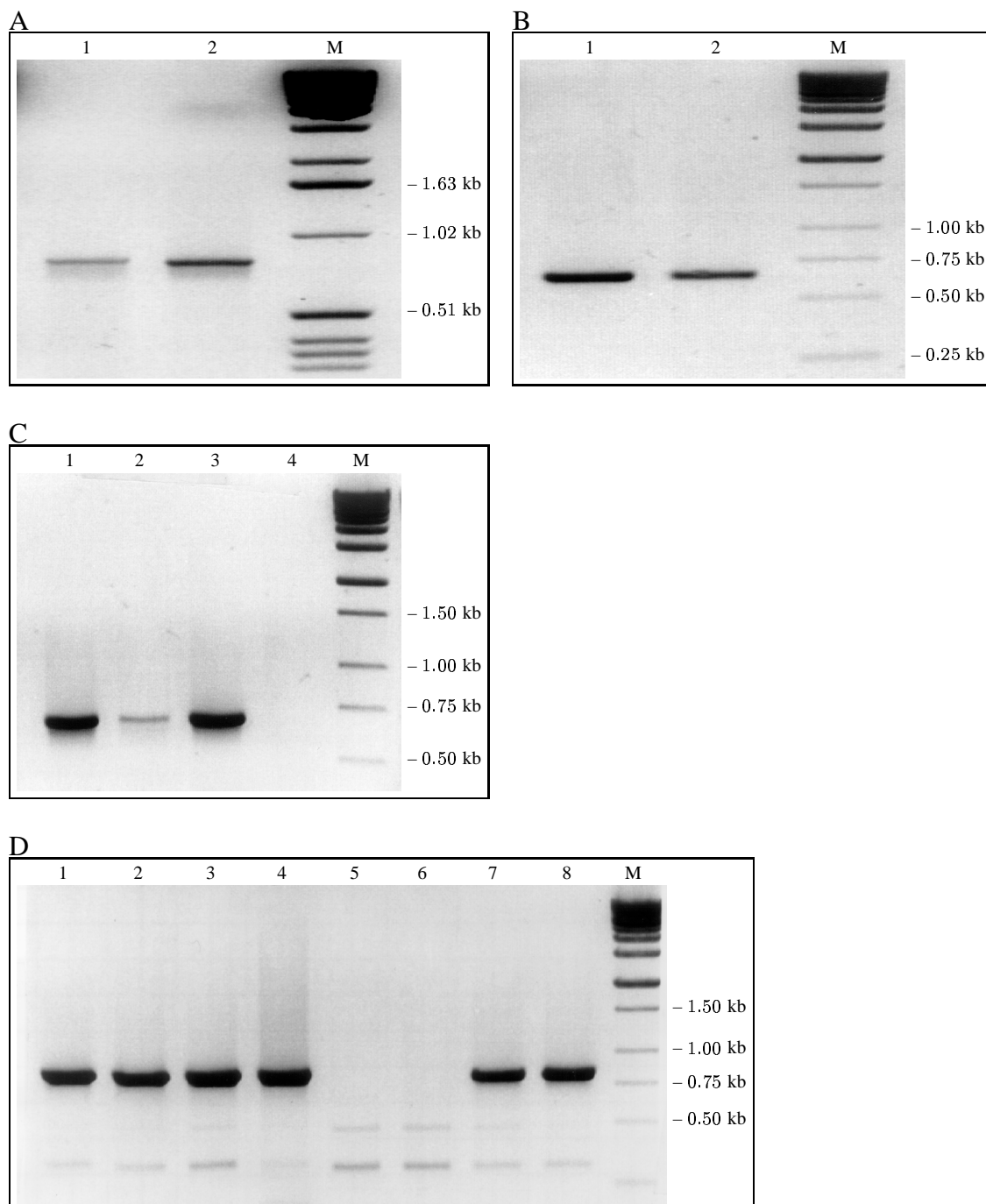
Figure 2.18: Construction of the *L. monocytogenes* single mutant strains $\Delta inlG$I, $\Delta inlG$II, $\Delta inlH$ and $\Delta inlE$. PCR analysis to isolate double cross-over mutants. Deletion of the genes *inlG* (A and B), *inlH* (C) and *inlE* (D) was detected by PCR with the primer pairs pGluco-1/inlYC2spez-1($\Delta inlG$I), PinlXu/lism51-24 ($\Delta inlG$II), PinlYu/delE-2 ($\Delta inlH$) and inlYseq-1/delxy-7 ($\Delta inlE$). As expected, the length of the obtained PCR products is 866, 647, 727 and 836 bp instead of 2234, 2021, 2230 and 2187 derived from the wild type sequences of *inlG*, *inlH* or *inlE*, respectively. Mutants were constructed in duplicate. PCR products of selected clones were sequenced (App. B.2).
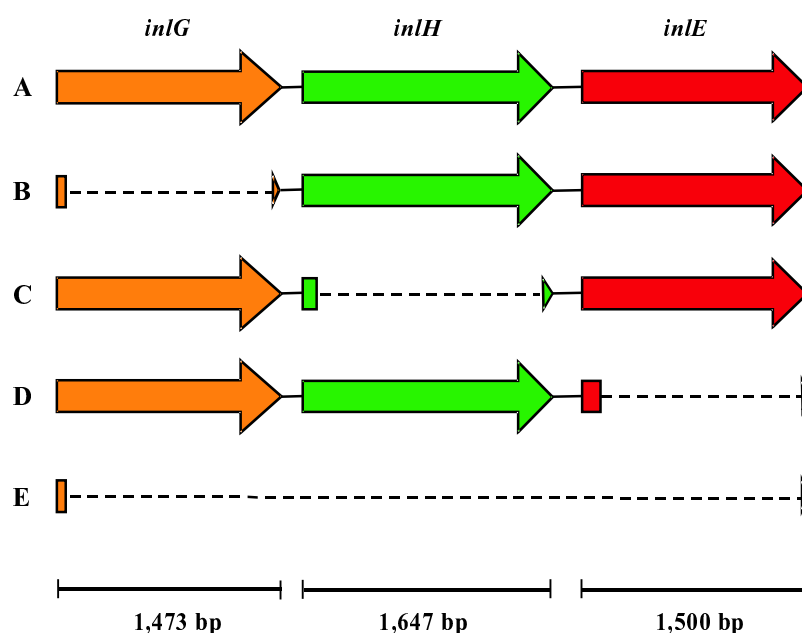
Figure 2.19: Genetic organization of the *inlGHE* gene cluster of *L. monocytogenes* in the wild type (A), Δ*inlG* (B), Δ*inlH* (C), Δ*inlE* (D) and Δ*inlGHE* (E) mutant strains. Genes are depicted as arrows. Deletions are represented by dashed lines. Length of the coding regions are indicated in base pairs (bp). Figure in scale.

by an intact copy of the *inlG* gene. Reversion of the Δ*inlE* mutant was performed in two different approaches using strain S40 in both cases. Revertant strains were constructed in duplicate and designated as S61 (*inlG*I$^+$), S62 and S63 (*inlG*II$^+$), S79 and S80 (*inlH*$^+$), S60 and S70 (*inlE*$^+$).

The revertant strains *inlG*II$^+$, *inlH*$^+$ and *inlE*$^+$ were tested for internalization into the microvascular endothelial cell line HBMEC. These cellular invasion assays were performed in a doctoral thesis by B. Bergmann from the University of Würzburg, Germany (Bergmann, Raffelsbauer *et al.*, manuscript submitted). As shown in Table 2.3, the revertant strains tested yielded invasion rates which are significantly lower than those of the single mutants. Thus, reversion of the Δ*inlG*II, Δ*inlH* and Δ*inlE* mutants by double cross-over with the corresponding wild type genes resulted in a decrease of invasiveness to a level which is comparable to that of the wild type strain, confirming that the enhanced invasion rates observed with the single mutants are specifically caused by the deletion of the internalin genes and can be reversed by restoring the wild type genotype.

2.11 Construction and characterization of *L. monocytogenes inl* combination mutants

It was previously shown that deletion of *inlA* or *inlB* reduces or even abolishes the ability of *L. monocytogenes* to invade mammalian cells *in vitro* (reviewed by Kuhn and Goebel,
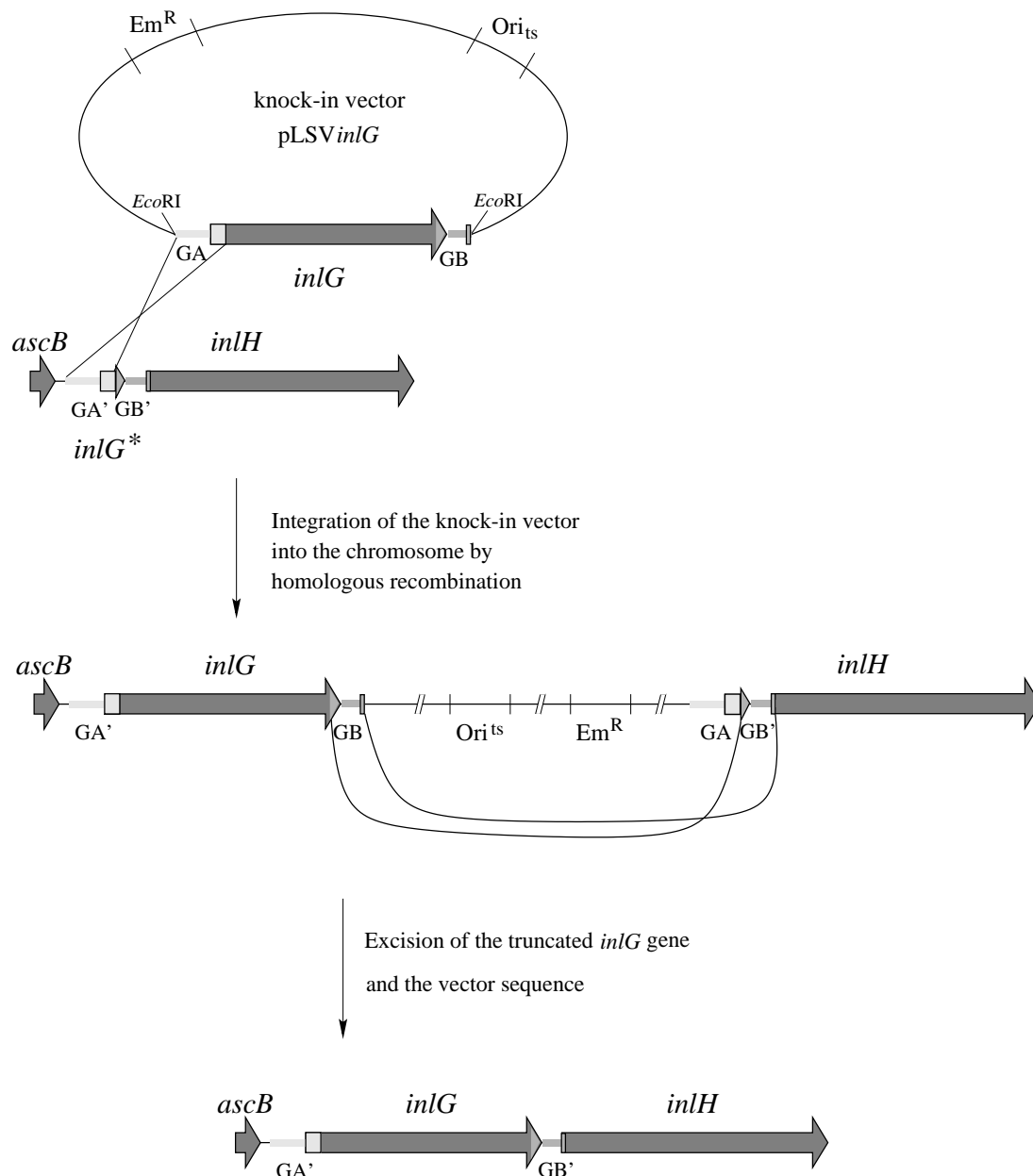
Figure 2.20: Strategy used for construction of the *L. monocytogenes* revertant strain *inlG*[+] by double cross-over using the knock-in vector pLSV*inlG*. *InlG* was amplified from the chromosome of *L. monocytogenes* wild type using the primers mutrevG-1 and lisminv-1. The amplified intact gene, which includes at the 5' and 3' ends the homology fragments GA and GB used to construct the Δ*inlG* mutant, was cloned into the vector pLSV1 via *Eco*RI restriction sites. Integration of the knock-in vector into the chromosome was induced by incubating bacteria at 42°C in the presence of erythromycin. Homologous recombination at the 5' end (between fragments GA and GA') was detected by PCR screening with appropriate primers. The second cross-over between fragments GB and GB' resulted in the excision of the truncated *inlG* gene and the intervening vector sequence from the chromosome, restoring the wild type *inlG* gene. Reversion was confirmed by PCR and nucleotide sequence analysis as shown in Fig. 2.22 and App. B.2.
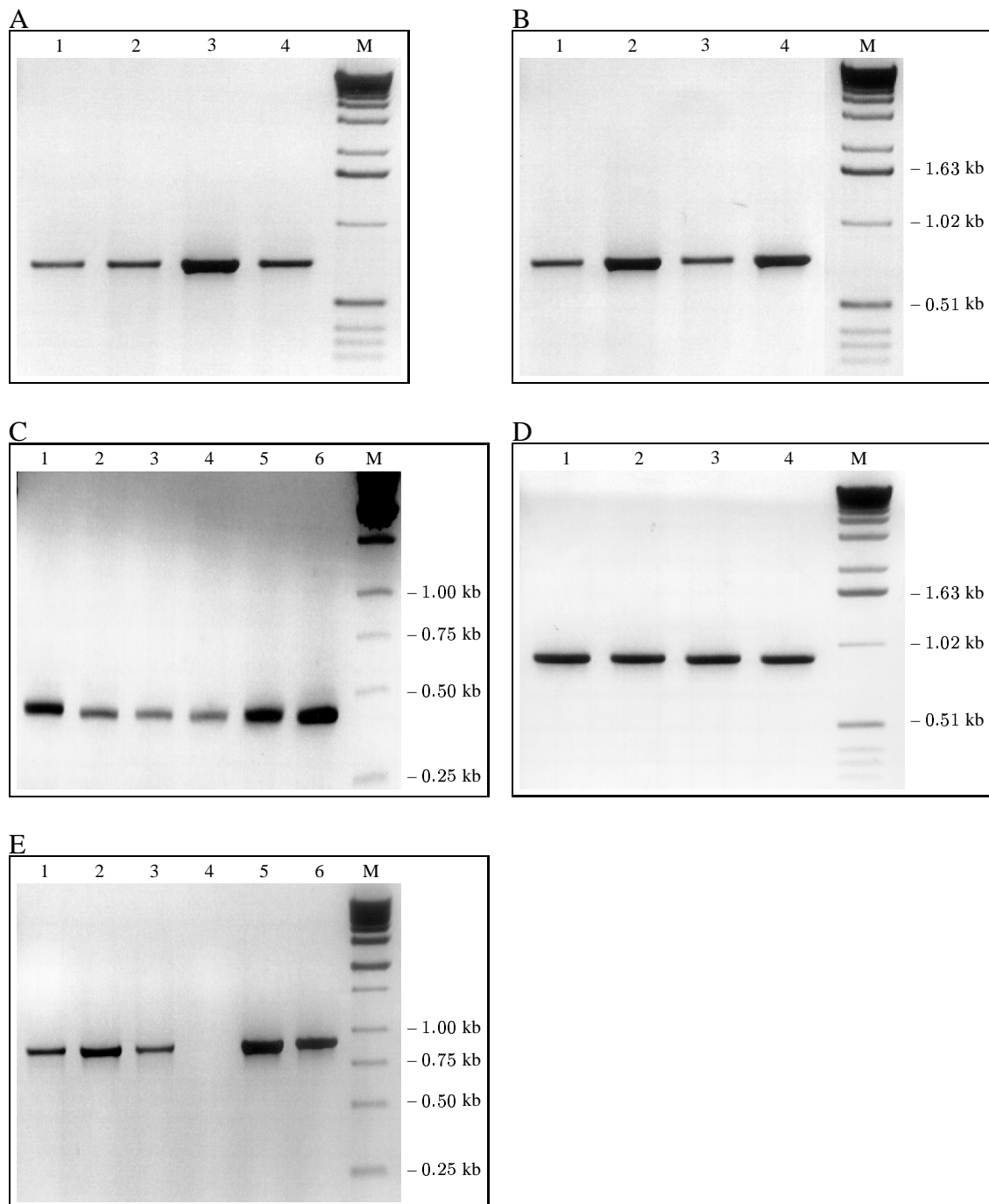
Figure 2.21: Construction of the *L. monocytogenes* revertant strains *inlG*$^+$, *inlH*$^+$ and *inlE*$^+$. PCR analysis to isolate single cross-over mutants. Integration of the knock-in plasmids pLSV*inlG* into the $\Delta inlG$I (A) and $\Delta inlG$II (B), of pLSV*inlH* into $\Delta inlH$ (C) and of pLSV*inlE* into $\Delta inlE$ (D and E) chromosome was detected by PCR using the primer pairs PinlXu/RTG7Bdown (*inlG*), PinlYu/inlYC2spez-1 (*inlH*) and inlYseq-1/inlZseq-3 (*inlE*), respectively. Note that both *inlG* deletion mutants $\Delta inlG$I and $\Delta inlG$II were reverted and that reversion of the $\Delta inlE$ mutant was performed in two different approaches.
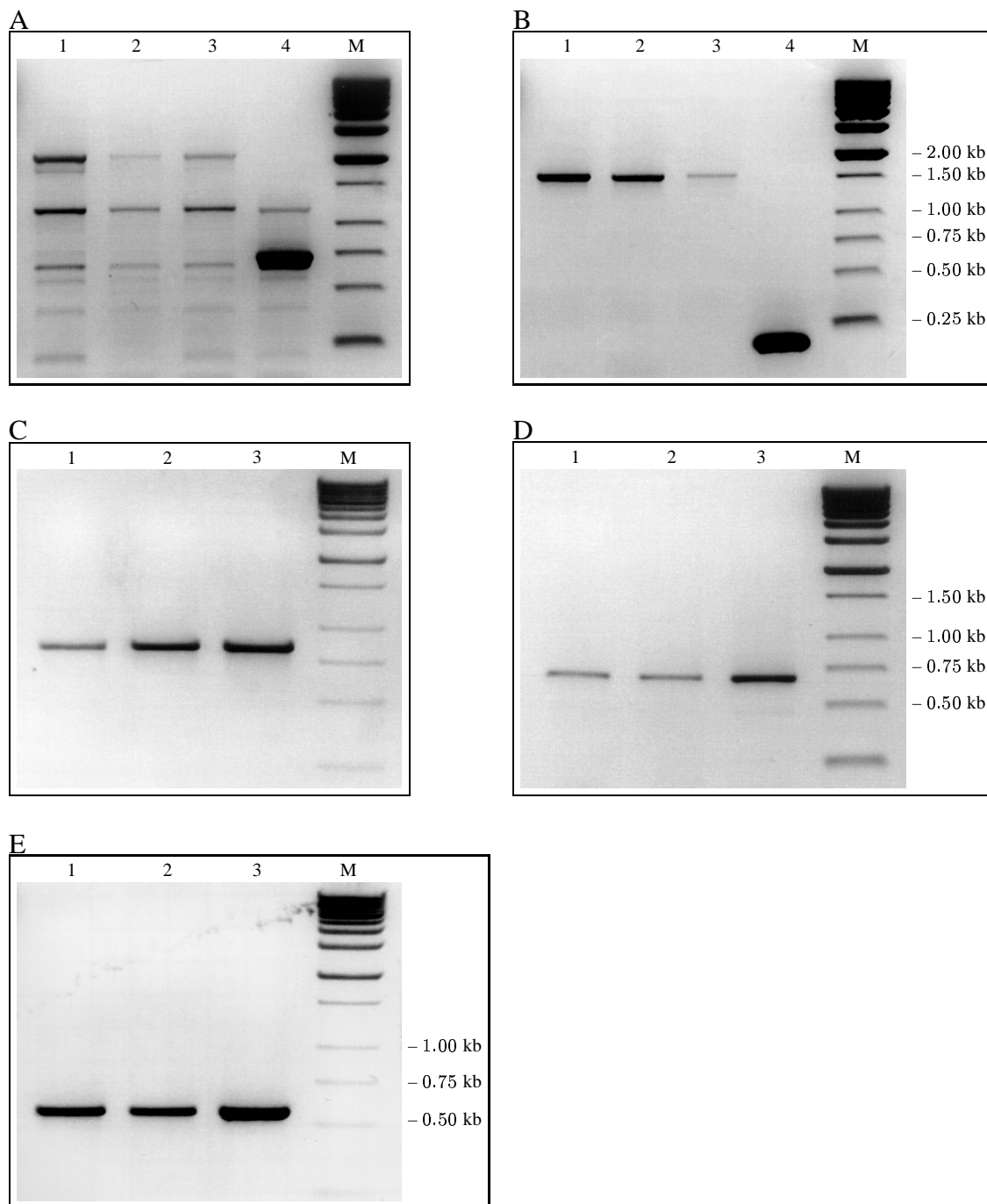
Figure 2.22: Construction of the *L. monocytogenes* revertant strains *inlG*$^+$. PCR analysis to isolate double cross-over mutants. Reversion of Δ*inlG*I and Δ*inlG*II by inserting a copy of the corresponding gene into the chromosome was verified by PCR screening using primers that yielded products spanning the complete gene at once (A and B) and additionally in smaller overlapping fragments (C, D and E). The long PCR products shown in A and B, which indicate the presence of the wild type *inlG* gene, were obtained with the primer pairs PinlXu/inlYC2spez-1 and RTG1up/inlX-1, yielding DNA fragments of 2,098 bp and 1,541 bp, respectively. Lanes 1, 2 and 3 correspond to the revertant strains S61, S62, S63, respectively. As control, the same reactions were performed using DNA from the single mutant Δ*inlG*II (A and B, lane 4).
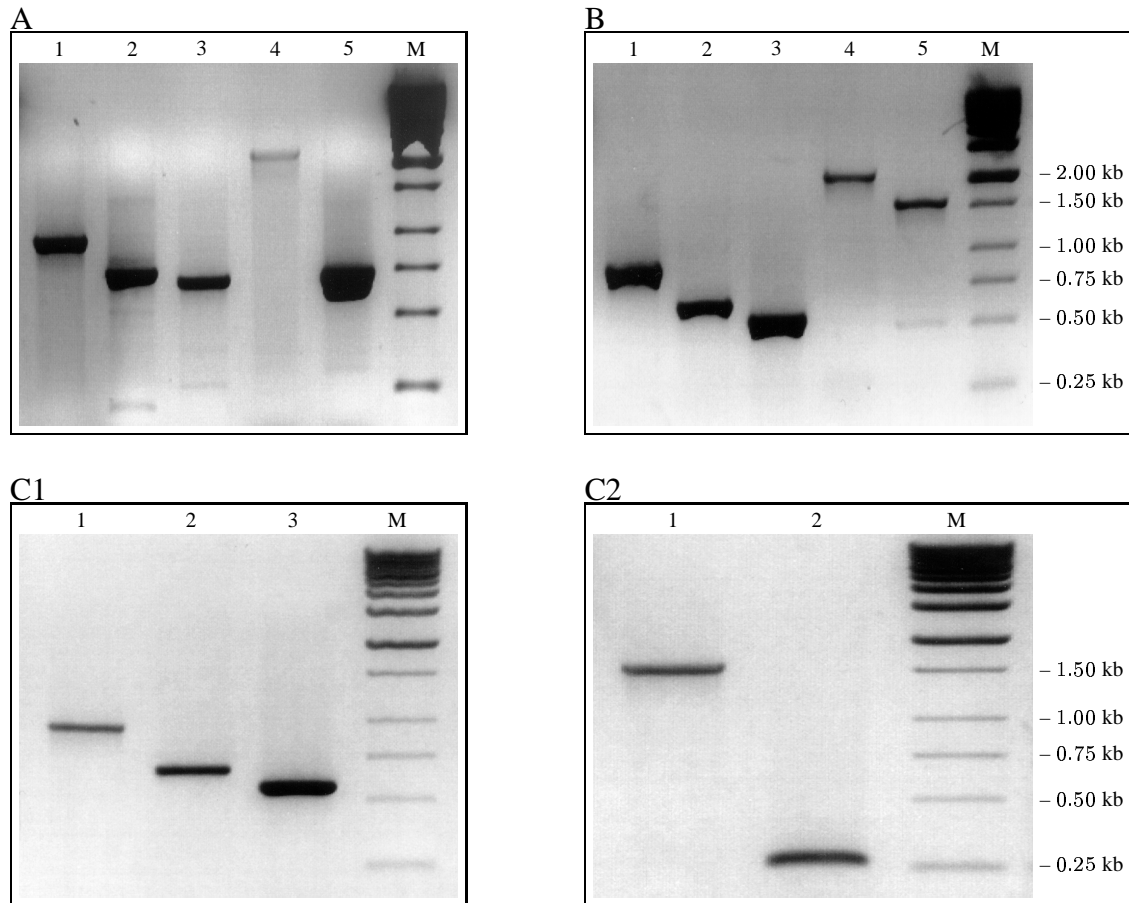
Figure 2.23: Construction of the *L. monocytogenes* revertant strains *inlH*⁺ and *inlE*⁺. PCR analysis to isolate double cross-over mutants. Reversion of the single mutants Δ*inlH* (A) and Δ*inlE* (B and C) by inserting a copy of the corresponding gene into the chromosome was verified by PCR screening using primers that yielded products spanning the complete gene in small overlapping fragments (A.1-3, B.1-3, C1.1-3) or at once (A.4, B.4-5, C2.1). The long PCR products indicating the presence of the wild type genes were obtained with the primer pairs PinlYu/delE-2 (*inlH*; A.4) and inlYseq-2/lism51-14 (*inlE*; B.5, C2.1), yielding DNA fragments of 2,230 and 1,618 bp, respectively. As control, the same reactions were performed using DNA from the single mutants Δ*inlH* and Δ*inlE* (A.5, C2.2). Revertants shown in A, B and C correspond to the strains S80, S70 and S60, respectively.

Table 2.3: Invasion of *L. monocytogenes* strains wild type, single deletion mutants Δ*inlG*II, Δ*inlH* and Δ*inlE*, and the corresponding revertants *inlG*II$^+$, *inlH*$^+$ and *inlE*$^+$ into HepG-2 cells and human brain microvascular endothelial cells (HBMEC). Data cited with kind permission of B. Bergmann (B. Bergmann, doctoral thesis and Bergmann, Raffelsbauer *et al.*, manuscript submitted).

HepG-2 cells		HBMEC	
Bacterial strain	Invasion rate	Bacterial strain	Invasion rate
Wild type	100 ± 10	Wild type	100 ± 23
Δ*inlGHE*	360 ± 32	Δ*inlGHE*	269 ± 31
Δ*inlG*II	306 ± 39	Δ*inlG*II	260 ± 50
Δ*inlH*	328 ± 36	Δ*inlH*	205 ± 33
Δ*inlE*	200 ± 23	Δ*inlE*	265 ± 50
		*inlG*II$^+$	159 ± 28
		inlH$^+$	79 ± 22
		inlE$^+$	66 ± 21

2000). Surprisingly, deletion of the *inlGHE* gene cluster leads rather to an increase in invasiveness of *L. monocytogenes* into several non-phagocytic cell lines, suggesting that these internalins, unlike InlA and InlB, do not directly act as invasins for internalization into mammalian cells, but seem to negatively interfere with this entry process. In order to test whether and how deletion of *inlGHE* in combination with other internalin genes affects invasiveness of *L. monocytogenes*, in the present study the *inlGHE* gene cluster was removed from mutants which already carried deletions in the genes *inlA*, *inlB* and/or *inlC*, thus creating the *inl* combination mutant strains Δ*inlA/GHE*, Δ*inlB/GHE*, Δ*inlC/GHE*, Δ*inlA/B/GHE* and Δ*inlB/C/GHE*. Deletion of *inlGHE* was achieved using the knock-out plasmid pLSVΔ*inlGHE*, which was also applied to construct the Δ*inlGHE* mutant strains as shown in Fig. 2.10 and described in detail in section 5.2. In addition, using the pLSVΔ*inlC* knock-out plasmid, the *inlC* gene was deleted from the strains Δ*inlA* and Δ*inlA/GHE*, thus creating the strains Δ*inlA/C* and Δ*inlA/C/GHE*. PLSVΔ*inlC* was constructed in the present study by cloning the truncated *inlC* gene which was amplified from the Δ*inlC* mutant (Engelbrecht *et al.*, 1996) into the pLSV1 vector. Integration of the knock-out plasmids and deletion of the genes were confirmed by PCR screening. The obtained PCR products are shown in Fig. 2.24, 2.25 and 2.26. The correct in-frame deletions were verified by PCR and DNA sequence analysis. The chromosomal regions comprising the deletions were amplified by PCR, cloned into the vector pUC18 and sequenced. Nucleotide sequences of the constructed *inl* combination deletion mutants are shown in App. B.2. Internalin combination mutants were constructed in duplicate and designated as follows: Δ*inlA/GHE* (S27 and S28), Δ*inlB/GHE* (S67 and S68), Δ*inlC/GHE* (S29 and S30), Δ*inlA/B/GHE* (S34 and S35), Δ*inlB/C/GHE* (S36), Δ*inlA/C* (S71 and S73) and Δ*inlA/C/GHE* (S75 and S76). A schematic representation showing the genetic organization of the genes *inlA*, *inlB* and *inlC* in the deletion mutants Δ*inlA*, Δ*inlB*, Δ*inlA/B* and Δ*inlC* compared to the wild type strain is depicted in Fig. 2.27.
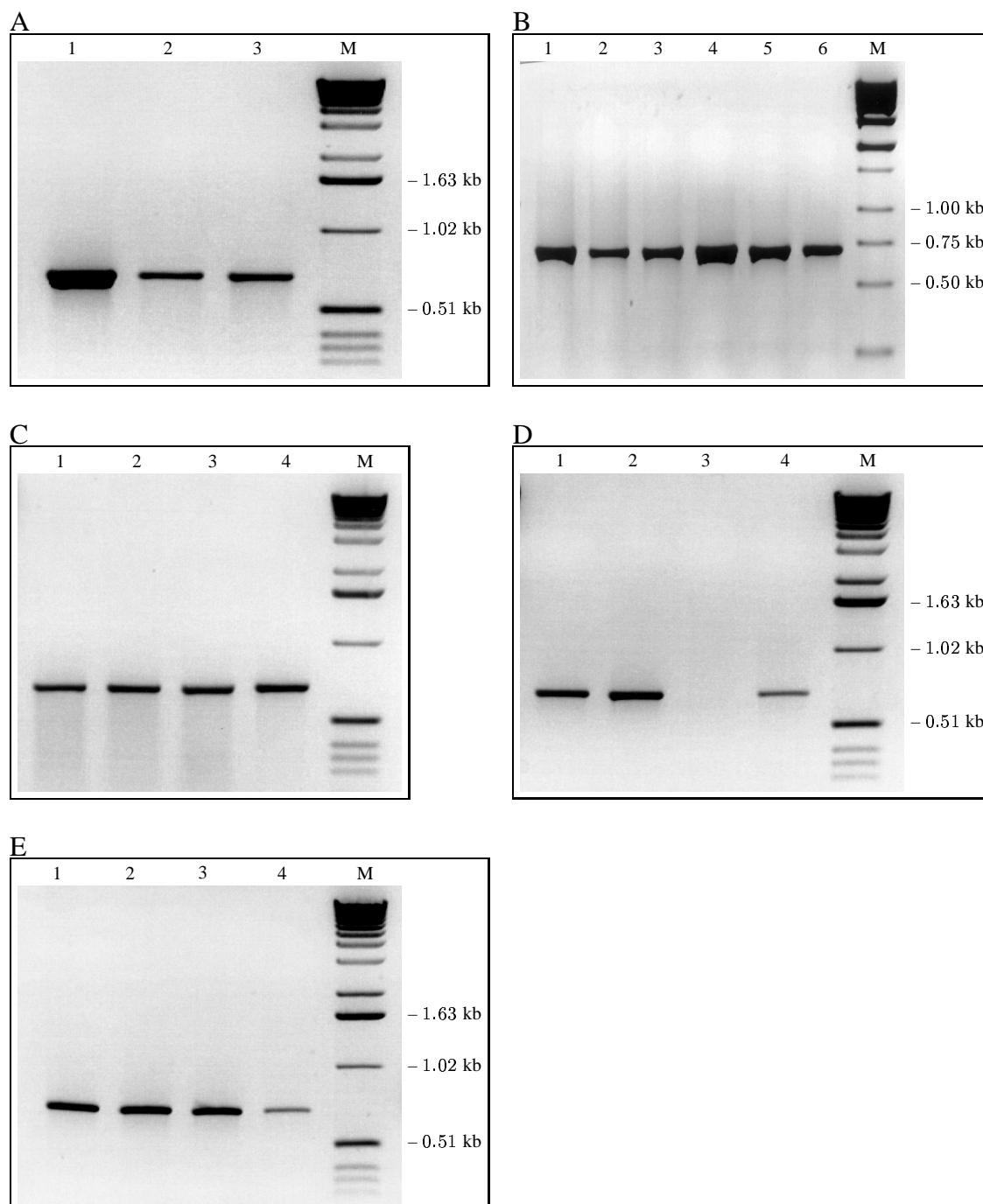
Figure 2.24: Construction of *L. monocytogenes inl* combination mutant strains. PCR analysis applied to isolate single cross-over mutants. Integration of the knock-out plasmid pLSV∆*inlGHE* into the chromosome of the *inl* deletion mutants ∆*inlA* (A), ∆*inlB* (B), ∆*inlC* (C), ∆*inlA/B* (D) and ∆*inlB/C* (E). Integration was detected by PCR using the primer pairs pGluco-1/ORFZ-1, yielding a PCR product of 703 bp.
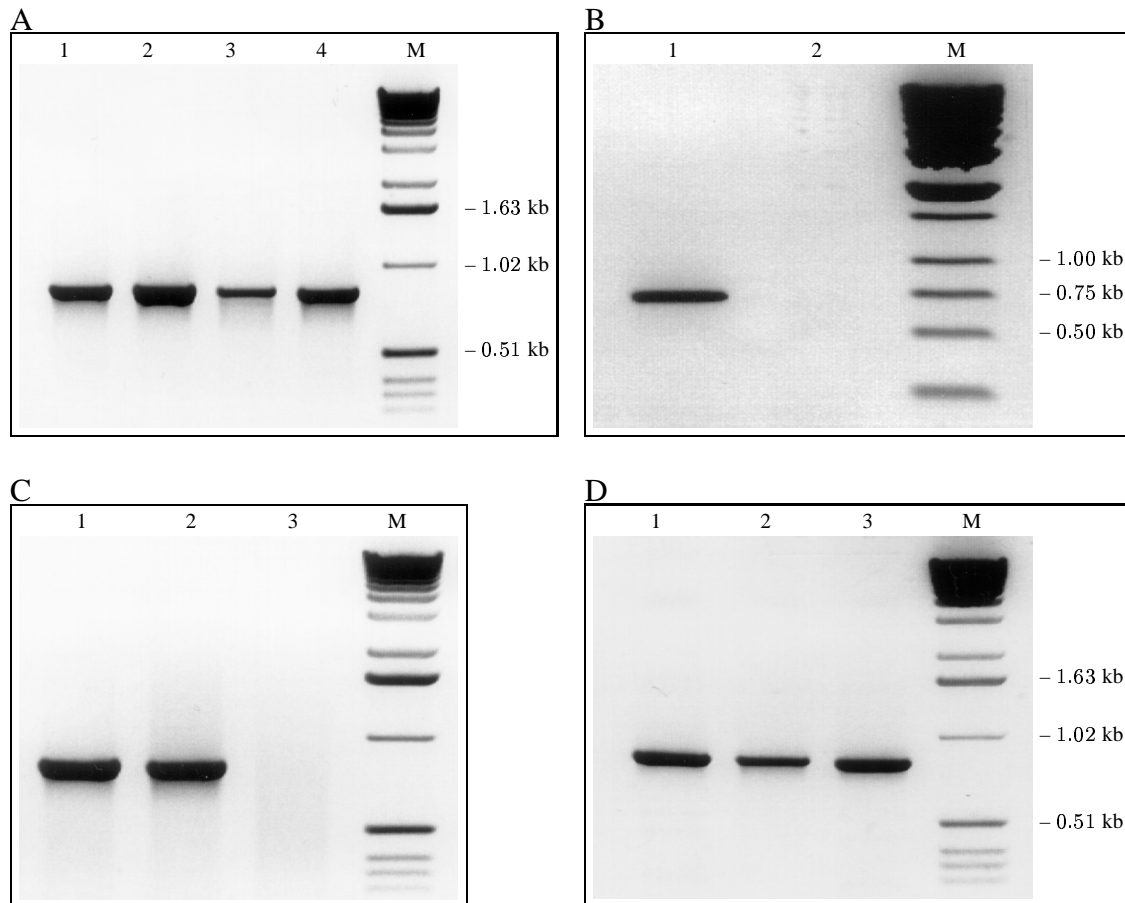
Figure 2.25: Construction of *L. monocytogenes inl* combination mutant strains. PCR analysis to isolate double cross-over mutants. Construction of the mutants Δ*inlA/GHE* (A), Δ*inlB/GHE* (B), Δ*inlC/GHE* (C), Δ*inlA/B/GHE* (D, lanes 1 and 2) and Δ*inlB/C/GHE* (D, lane 3). Deletion of the *inlGHE* gene cluster was detected by PCR using the primers PinlXu and delxy-7, which yielded a shortened PCR product of 728 bp instead of 5,602 bp from the wild type sequence. PCR products shown were sequenced. Nucleotide sequences of the *inl* combination mutants Δ*inlA/GHE*, Δ*inlB/GHE*, Δ*inlC/GHE*, Δ*inlA/B/GHE*, Δ*inlB/C/GHE* are shown in App. B.2.
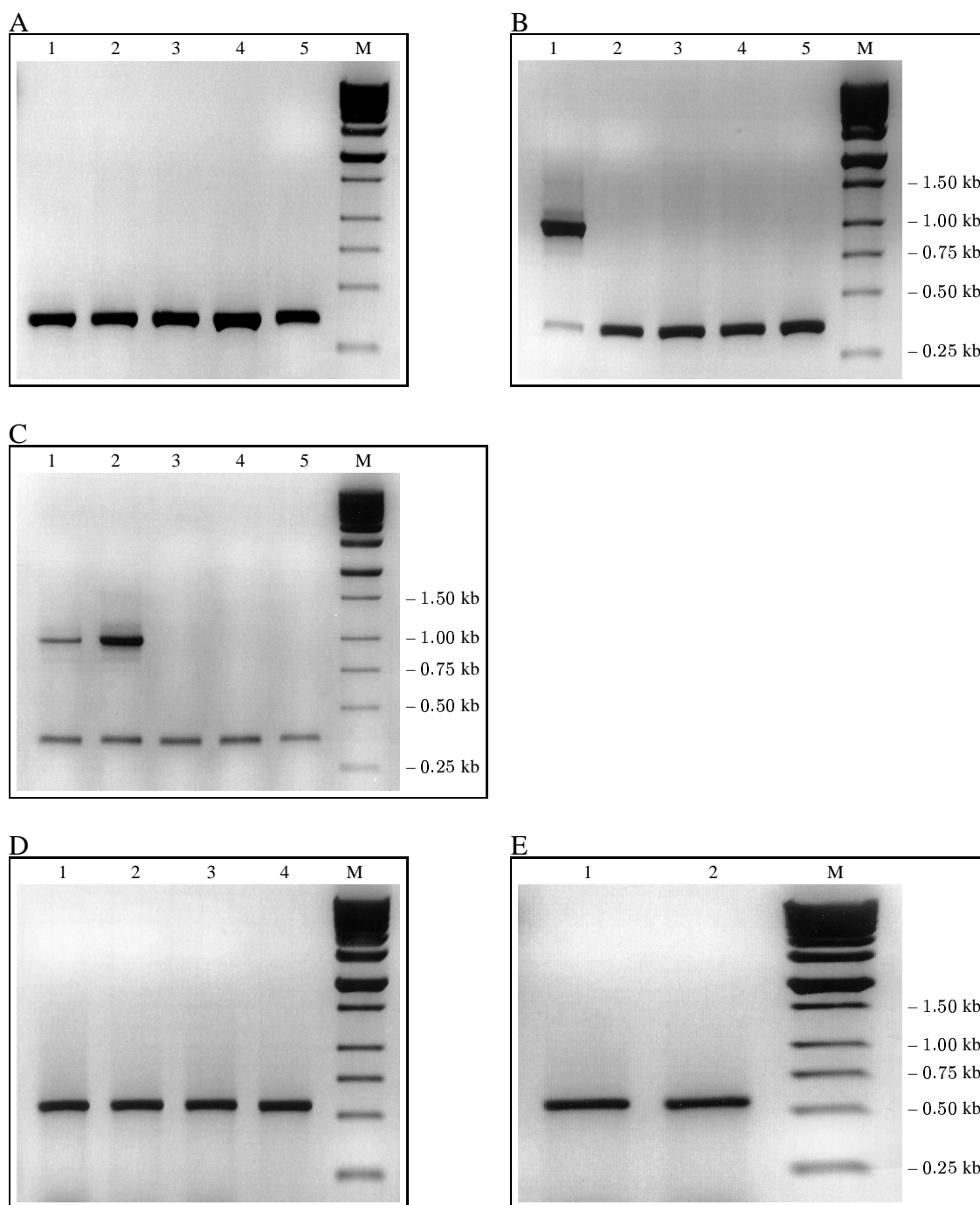
Figure 2.26: Construction of *L. monocytogenes inl* combination mutant strains. PCR analysis to isolate single and double cross-over mutants. A-C. Integration of the knock-out plasmid pLSV∆*inlC* into the chromosome of the *inl* deletion mutants ∆*inlA* (A), ∆*inlA*/*GHE* (B) and ∆*inlGHE* (C). Integration was detected by PCR using the primer pair InlC-3/delCseq-2, yielding a PCR product of 362 bp. D-E. Construction of the double cross-over mutants ∆*inlA*/*C* (D) and ∆*inlA*/*C*/*GHE* (E). Deletion of *inlC* was verified with the primers InlC-3 and InlC-4. The obtained product was 578 bp long, in contrast to the 1,229 bp long fragment derived from the wild type sequence. PCR products shown in D and E were sequenced. Nucleotide sequences of the *inl* combination mutants ∆*inlA*/*C* and ∆*inlA*/*C*/*GHE* are shown in App. B.2.
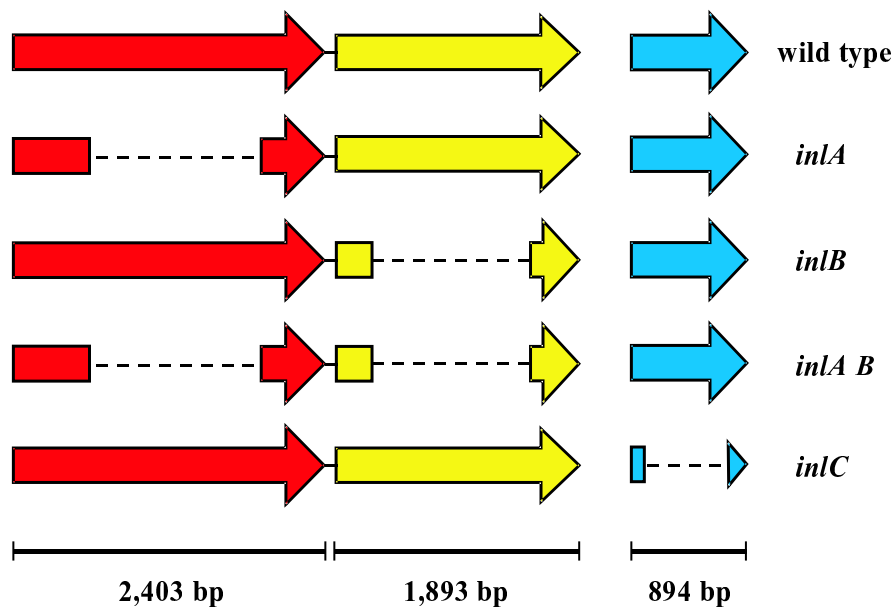
Figure 2.27: Schematic representation of the genes *inlA*, *inlB* and *inlC* in the deletion mutants Δ*inlA*, Δ*inlB*, Δ*inlA/B* and Δ*inlC* compared to the wild type strain. Genes are depicted as arrows. Deletions are represented by dashed lines. Length of the coding regions are indicated in base pairs (bp). Figure in scale.

To determine a putative contribution of the internalins InlG, InlH and InlE to the entry of *L. monocytogenes* into mammalian cells, the *inl* combination mutants lacking the *inl-GHE* gene cluster constructed in the present work were tested together with the wild type strain, the Δ*inlGHE* mutant and the corresponding parental mutant strains Δ*inlA*, Δ*inlB*, Δ*inlC*, Δ*inlA/B*, Δ*inlB/C* and Δ*inlA/C* for their capacity to invade the non-phagocytic cell lines HBMEC and Caco-2. These cellular invasion assays were performed in a doctoral thesis by B. Bergmann from the University of Würzburg, Germany (Bergmann, Raffelsbauer *et al.*, manuscript submitted). As shown in Table 2.4 all mutants lacking the *inlB* gene were impaired to invade cells of the HBMEC cell line, confirming previous studies which showed that internalization of *L. monocytogenes* into HBMEC depends exclusively on InlB (Greiffenberg *et al.*, 1998). Deletion of *inl* genes other than *inlB* does not affect invasion ability, as mutants lacking *inlA*, *inlC* and/or *inlGHE* are still invasive for HBMEC, showing that this InlB-mediated invasion does not require additional internalins. Interestingly, deletion of *inlGHE* alone or in combination with other *inl* genes leads to a two- to three-fold increase in invasiveness into HBMEC in all mutants constructed, except in strain Δ*inlC/GHE*. These data indicate that the presence of *inlGHE* inhibits InlB-mediated invasion and suggest that these internalins might compete for the same receptor on the cell surface (Bergmann, Raffelsbauer *et al.*, manuscript submitted).

Entry of *L. monocytogenes* into Caco-2 cells was shown to require mainly InlA (Gaillard *et al.*, 1991; Dramsi *et al.*, 1995; Mengaud *et al.*, 1996), but also to some extent InlB (Lingnau *et al.*, 1995). As shown in Table 2.5, this previous observation was confirmed,

since deletion of *inlA* or *inlB* reduces invasiveness to a very low level, and deletion of both genes together impairs *L. monocytogenes* to enter Caco-2 cells at all (Bergmann, Raffelsbauer *et al*., manuscript submitted). In the absence of *inlB*, the additional deletion of *inlGHE* or especially of *inlC* leads to a significant reduction in invasiveness. The mutants Δ*inlB*/*C* and Δ*inlB*/*C*/*GHE* are nearly non-invasive for Caco-2 cells, although these strains carry an intact *inlA* gene. However, invasiveness of these mutants is restored in the presence of *inlB*, as the mutants Δ*inlC* and Δ*inlC*/*GHE* show invasion rates similar to those of the wild type strain. Taken together, these data indicate that the InlA-mediated invasion into Caco-2 cells requires the function of InlB, InlC, InlG, InlH and InlE (Bergmann, Raffelsbauer *et al*., manuscript submitted).

Table 2.4: Invasion of *L. monocytogenes* internalin deletion mutants into human brain microvascular endothelial cells (HBMEC). Data cited with kind permission of B. Bergmann (B. Bergmann, doctoral thesis and Bergmann, Raffelsbauer *et al*., manuscript submitted).

Bacterial strain	Invasion rate	Bacterial strain	Invasion rate
Wild type	100 ± 13	Δ*inlGHE*	294 ± 24
Δ*inlA*	91 ± 6	Δ*inlA*/*GHE*	141 ± 31
Δ*inlC*	86 ± 26	Δ*inlC*/*GHE*	80 ± 13
Δ*inlA*/*C*	82 ± 12	Δ*inlA*/*C*/*GHE*	248 ± 30
Δ*inlB*	0	Δ*inlB*/*GHE*	0
Δ*inlA*/*B*	0	Δ*inlA*/*B*/*GHE*	0
Δ*inlB*/*C*	0	Δ*inlB*/*C*/*GHE*	0

Table 2.5: Invasion of *L. monocytogenes* internalin deletion mutants into Caco-2 cells. Data cited with kind permission of B. Bergmann (B. Bergmann, doctoral thesis and Bergmann, Raffelsbauer *et al*., manuscript submitted).

Bacterial strain	Invasion rate	Bacterial strain	Invasion rate
Wild type	100 ± 23	Δ*inlGHE*	310 ± 9
Δ*inlA*	12 ± 4	Δ*inlA*/*GHE*	19 ± 11
Δ*inlC*	88 ± 5	Δ*inlC*/*GHE*	60 ± 14
Δ*inlA*/*C*	10 ± 5	Δ*inlA*/*C*/*GHE*	23 ± 2
Δ*inlB*	36 ± 2	Δ*inlB*/*GHE*	21 ± 2
Δ*inlA*/*B*	0	Δ*inlA*/*B*/*GHE*	0
Δ*inlB*/*C*	2 ± 1	Δ*inlB*/*C*/*GHE*	2 ± 2

2.12 Deletion of *inlGHE* enhances adhesion of *L. monocytogenes* to differentiated Caco-2 cells

To examine whether the enhanced invasiveness observed with the *inlGHE* deletion mutant is caused by an increase in the ability of this mutant to adhere to mammalian cells, adhesion assays were performed using confluent monolayers of Caco-2 cells which showed fully differentiated cell morphology. These experiments were performed by Prof. Dr. I. Karunasagar at the University of Würzburg, Germany. As listed in Table 2.6, the number of Δ*inlGHE* mutant bacteria which adhered to these Caco-2 cells was three- to four-fold higher than that of the wild type strain. A similar increase was also observed in the invasiveness of the adherent mutant bacteria, leading to a total invasion rate which was considerably higher than that of the wild type. Whereas adherence of the Δ*inlB* mutant was not affected, the numbers of adherent Δ*inlA* and Δ*inlC* bacteria were considerably reduced (approx. 20% and 60%, respectively, compared to the parental strain), suggesting that these genes are involved in adhesion to Caco-2 cells. Interestingly, relative to the number of adherent bacteria, all three *inl* mutants showed invasion rates which were similar to that of the wild type, indicating that deletion of the genes *inlA*, *inlB* and *inlC* does not affect invasion into confluent, differentiated Caco-2 cells. In addition, the Δ*inlA/C* mutant constructed in the present work was nearly impaired to adhere to these cells (Karunasagar, personal communication), suggesting that both InlA and InlC proteins may act together to mediate efficient adhesion (Karunasagar and Raffelsbauer, unpublished data).

Table 2.6: Adhesion and invasion of *L. monocytogenes* internalin deletion mutants into Caco-2 cells (confluent monolayers). Adherence rate was calculated relative to the inoculum. Invasion rate was determined relative to the number of adherent bacteria. Data cited with kind permission of Dr. I. Karunasagar (Karunasagar and Raffelsbauer, unpublished data).

L. monocytogenes strain	Adherence rate	Invasion rate of adherent bacteria
Wild type	0.30	1.9
Δ*inlGHE*	1.10	8.2
Δ*inlA*	0.05	1.8
Δ*inlB*	0.28	1.9
Δ*inlC*	0.19	1.8

The results obtained by plating out adherent viable bacteria were confirmed by scanning electron microscopy, since more bacteria of the Δ*inlGHE* mutant than of the wild type strain were seen firmly attached to the apical side of the Caco-2 monolayer (data not shown). In contrast, only few bacteria of the Δ*inlA* and Δ*inlA/C* mutants could be seen, suggesting that InlA, either alone or together with InlC, mediates adhesion to differentiated Caco-2 cells (Karunasagar and Raffelsbauer, unpublished data). In general, no significant difference in the way bacteria adhered to cells was observed between the inter-

nalin mutants and the wild type strain. These scanning electron microscopic studies were
performed by Prof. Dr. I. Karunasagar in a cooperation work with Prof. Dr. G. Krohne at
the University of Würzburg, Germany.

2.13 Transcriptional studies of the genes *inlA*, *inlB* and *inlC* using the semi-quantitative RT-PCR technique

In order to investigate whether the altered invasiveness of the *inl* deletion mutants stud-
ied above is caused by a modified expression of the internalin genes, which in turn may
influence the bacterial uptake into non-phagocytic cells, transcription of the *inlA*, *inlB*
and *inlC* genes in these mutants in comparison to the wild type strain was studied using
a semi-quantitative RT-PCR assay. *L. monocytogenes* strains were grown in BHI to the
logarithmic phase and RNA was isolated as recently described (Dietrich *et al.*, 2000). To
compare different preparations, equivalent numbers of bacteria and equal quantities of
total RNA were used for reverse transcription (RT). To guarantee the same RT efficiency
for all genes tested, the *inlA*, *inlB* and *inlC* genes were reverse transcribed together in one
RT reaction. In addition, the *sod* gene, which encodes the superoxide dismutase and is
constitutively expressed under extracellular growth conditions, was used as internal stan-
dard. For RT-PCRs, gene-specific primers were designed after computer analysis of the
nucleotide sequences. The specificity of the primers was verified in control PCRs us-
ing chromosomal DNA as template, which yielded single PCR products of the expected
sizes. All RNA preparations were free of DNA, as shown by PCR performed prior to
the reverse transcription (Fig. 2.28). Isolated RNAs were reverse transcribed using the
moloney murine leukemia virus reverse transcriptase and 5 μg total RNA as template.
The generated cDNAs were diluted in exponential steps from 1:2 to 1:2048 and different
dilutions were tested for optimal PCR conditions as shown in Fig. 2.29. For subsequent
amplifications by PCR the highest dilutions of the cDNAs which still yielded visible in-
saturated PCR products were used. Since the genes tested are transcribed at different
levels, the optimal dilutions were 1:32 for *inlA* and *inlC*, 1:8 for *inlB* and 1:1024 for *sod*.
At least three independent PCRs were performed per sample.

2.13.1 Deletion of *inlGHE* alters expression of the *inlA* and *inlB* genes

The RT-PCR technique applied in this study demonstrated that there are differences in the
level of transcripts between the internalin mutants and the wild type strain. Deletion of
inlGHE and *inlB* either alone or in combination with each other induces transcription of
the *inlA* gene. As shown in Fig. 2.30, higher amounts of the *inlA* transcript could be de-
tected in the mutant strains lacking *inlGHE* and/or *inlB* compared to the wild type strain.
On the other hand, deletion of single genes from the *inlGHE* gene cluster apparently did
not affect transcription of *inlA*. A similar effect was observed when transcription of *inlB*
was examined. The levels of the *inlB* transcript were higher in the Δ*inlGHE*, Δ*inlA*,
Δ*inlA*/*GHE*, Δ*inlA*/*C* and Δ*inlA*/*C*/*GHE* mutant strains than in the wild type. Interest-
ingly, no increase of this transcript could be detected in the Δ*inlC* and Δ*inlC*/*GHE* mu-
tants. Again, the amounts of the *inlB* transcript in the single mutants Δ*inlG*, Δ*inlH* and
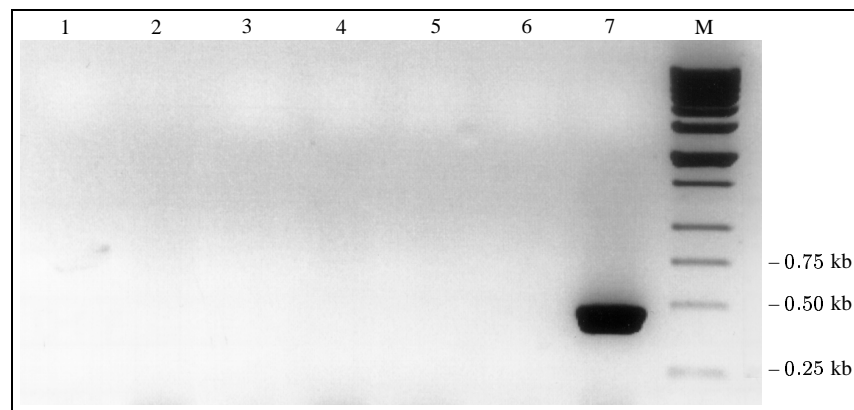
Figure 2.28: PCR analysis using RNA isolated from different *L. monocytogenes* strains after digestion of DNA with RNase-free DNase prior to reverse transcription. PCR was performed using primers the SOD-N and SOD-C derived from the *sod* gene. None of the aliquots yielded a PCR product, indicating that DNA was completely digested. As positive control, chromosomal DNA isolated from *L. monocytogenes* EGD wild type was used (lane 7).

$\Delta inlE$, and in the revertant strain $inlG^+$ were similar to that of the wild type. In contrast to the *inlA* and *inlB* genes, no significant difference in the transcription pattern of the *inlC* gene was observed between the internalin deletion mutants and the wild type. Similarly, the control RT-PCRs using the *sod* primers yielded the same amount of PCR products in the strains tested, indicating that similar quantities of total RNA were used in this assay. Therefore, differences observed in the amount of PCR products obtained with the *inlA* and *inlB* specific primers are caused by different levels of transcripts of these internalin genes in the mutant strains in comparison to the wild type.

Next it was examined whether the increased transcription of the genes *inlA* and *inlB* observed in the $\Delta inlGHE$ mutant during the mid-log phase could also be detected at different time points of the growth curve. The $\Delta inlGHE$ mutant and wild type strains were grown in BHI to the optical densities 0.5, 1.0 and 1.4 at 550 nm, which correspond to the early-, mid- and late-logarithmic phases, respectively. At these time points, equivalent numbers of bacteria were harvested. To remove remaining DNA, RNA aliquots were treated with RNase-free DNase and the complete DNA digestion was confirmed by PCR using samples of the RNA aliquots prior to the reverse transcription as template (Fig. 2.31). Isolated RNAs were reverse transcribed using 5 μg total RNA as template, and for subsequent PCR amplifications cDNAs were used in the dilutions described above. As shown in Fig. 2.32, higher levels of both *inlA* and *inlB* transcripts were detected in the $\Delta inlGHE$ mutant at all three time points chosen, indicating that the enhanced transcription of these genes is not transient but continuous along the logarithmic phase of bacterial growth. Again, no difference in transcription of *inlC* or the standard gene *sod* was observed. Interestingly, a slight increase in transcription of *inlC* in the late log phase compared to the early log phase could be detected in both strains.
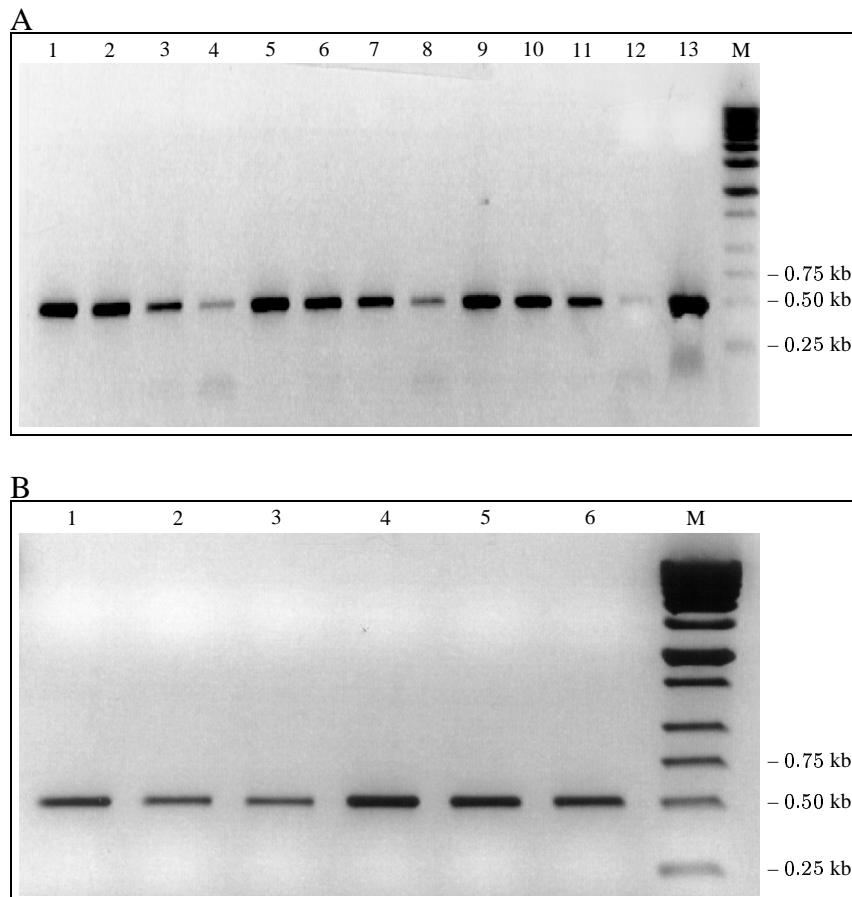
Figure 2.29: PCRs using primers derived from *inlA* (A, lanes 1-12), *inlB* (B, 1-3) and *inlC* (B, 4-6) to test for optimal dilutions of cDNAs from *L. monocytogenes* wild type. RNA was isolated at optical densities of 0.5 (A, 1-4), 1.0 (A, 5-8; B, 1-6) and 1.4 (A, 9-12) and reverse transcribed as described in section 5.3.2. A. The cDNAs were diluted 1:8 (1, 5, 9), 1:16 (2, 6, 10), 1:32 (3, 7, 11) and 1:64 (4, 8, 12). B. The cDNAs were used in the dilutions 1:8 (1, 4), 1:16 (2, 5) and 1:32 (3, 6).
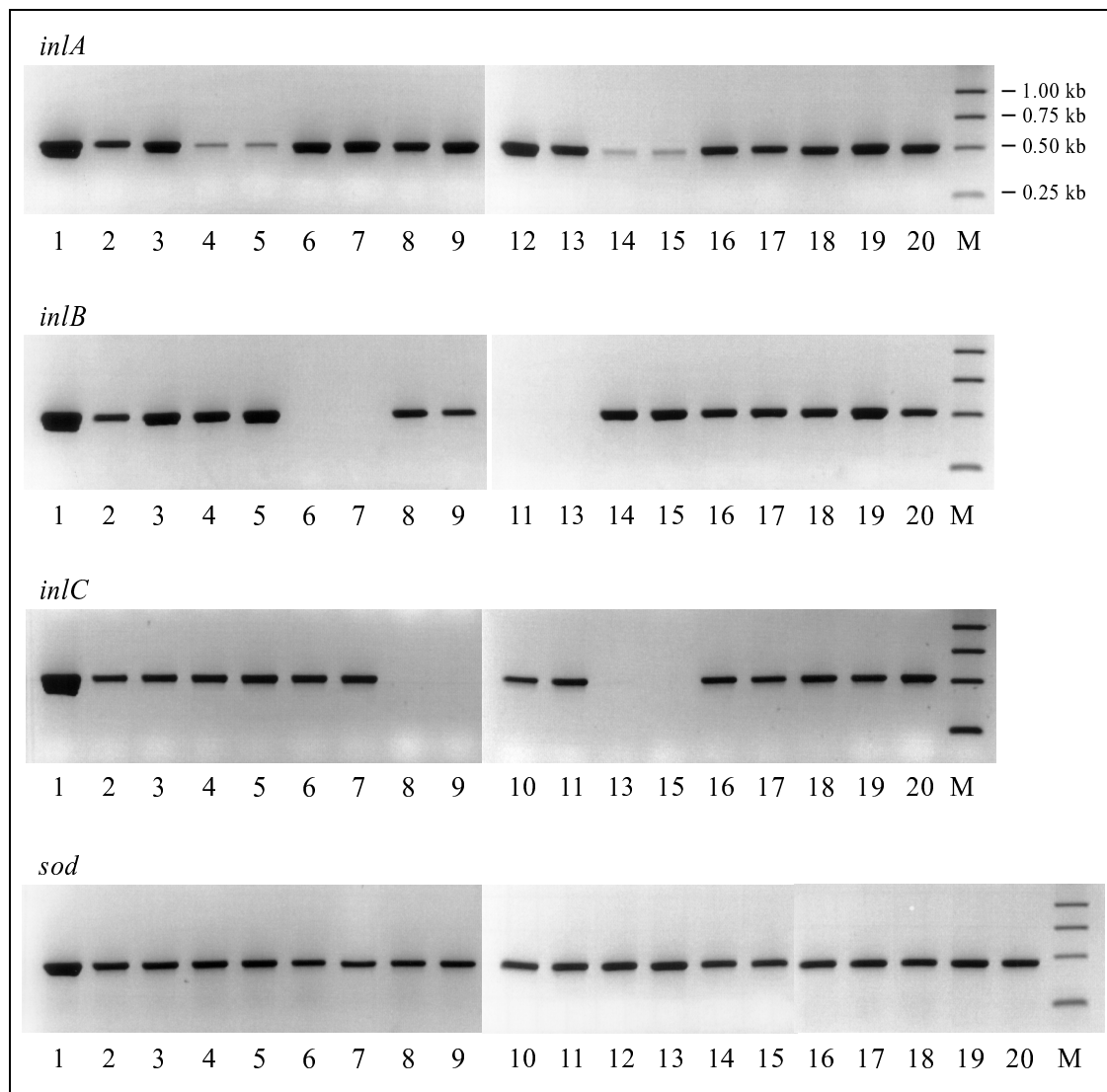
Figure 2.30: Comparison by semi-quantitative RT-PCRs of the transcription of the genes *inlA*, *inlB*, *inlC* and *sod* in the *L. monocytogenes* wild type and internalin deletion mutant strains. Bacteria were grown in BHI to the mid-log phase. The cDNAs were prepared from 5 μg total RNA and used in subsequent amplifications by PCR in the following dilutions: 1:32 for *inlA* and *inlC*, 1:8 for *inlB* and 1:1024 for *sod*. PCR products of *inlA* (515 bp), *inlB* (525 bp), *inlC* (532 bp) and *sod* (471 bp) were obtained with the primer pairs RTA1up/RTA2down, RTB1up/RTB2down, RTC1up/RTC2down and SOD-N/SOD-C, respectively. The DNA fragments were amplified from the 5' end of the coding region of the corresponding genes. 20 from 100 μl total volume of each PCR were loaded on agarose gels. Gels photographed with the same intensity were matched by comparing either single bands of the DNA molecular weight standard or PCR products from the wild type. Lanes: Control PCR with chromosomal DNA (lane 1), RT-PCRs of *L. monocytogenes* strains wild type (lane 2), $\Delta inlGHE$ (3), $\Delta inlA$ (4), $\Delta inlA/GHE$ (5), $\Delta inlB$ (6), $\Delta inlB/GHE$ (7), $\Delta inlC$ (8), $\Delta inlC/GHE$ (9), $\Delta inlA/B$ (10), $\Delta inlA/B/GHE$ (11), $\Delta inlB/C$ (12), $\Delta inlB/C/GHE$ (13), $\Delta inlA/C$ (14), $\Delta inlA/C/GHE$ (15), wild type (16), $\Delta inlG$ (17), $\Delta inlH$ (18), $\Delta inlE$ (19) and $inlG^+$ (20).
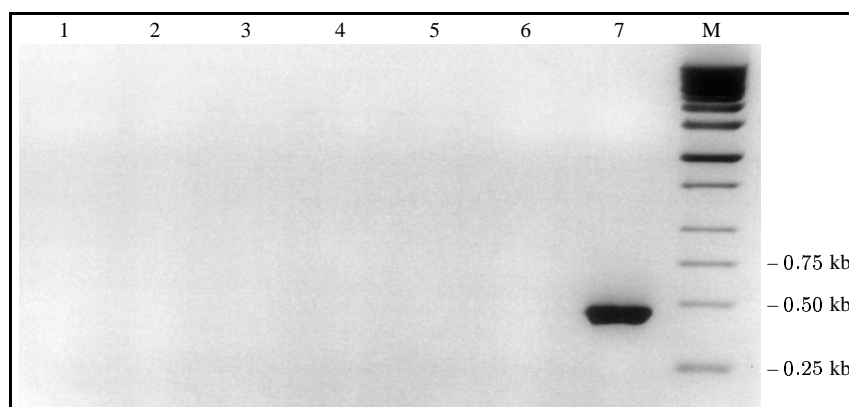
Figure 2.31: PCR analysis using RNA isolated from *L. monocytogenes* strains wild type (lanes 1, 3 and 5) and Δ*inlGHE* (2, 4 and 6) in the early-, mid- and late-logarithmic phases, respectively, after digestion of DNA with RNase-free DNase prior to reverse transcription. PCR was performed using the primers SOD-N and SOD-C derived from the *sod* gene. None of the aliquots yielded a PCR product, indicating that DNA was completely digested. As positive control, chromosomal DNA of the wild type strain was used (lane 7).
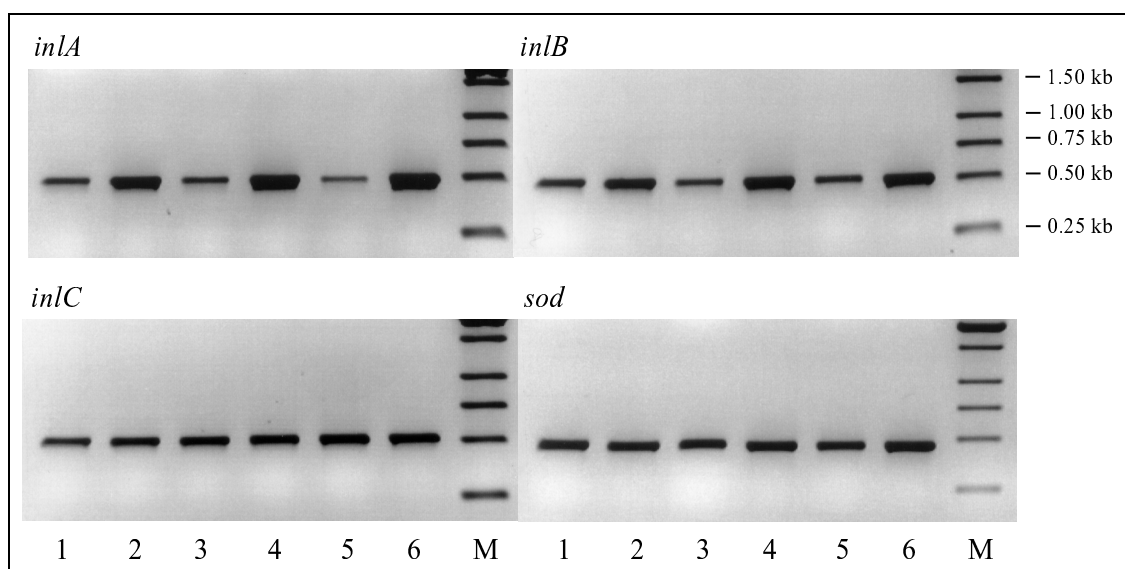
Figure 2.32: Comparison by semi-quantitative RT-PCRs of the transcription of the genes *inlA*, *inlB*, *inlC* and *sod* in the *L. monocytogenes* strains wild type (lanes 1, 3 and 5) and Δ*inlGHE* (lanes 2, 4 and 6) in the early (1 and 2), mid (3 and 4) and late (5 and 6) logarithmic phases of bacterial growth. The cDNAs were prepared and amplified as described above. 20 from 100 μl total volume of each PCR were loaded on agarose gels.

2.14 Transcriptional studies of the *inlGHE* gene cluster using the semi-quantitative RT-PCR technique

The transcriptional studies of the *inlGHE* gene cluster performed using the GFP expression plasmids as reporter showed that the *inlG* promoter is the most active promoter of this cluster, but lower activities of the *inlH* and *inlE* promoters were also detected (Fig. 2.9). However, these studies did not examine whether *inlH* and *inlE* are transcribed only by their own promoters or additionally as polycistronic transcripts starting from the *inlG* promoter. Therefore, transcription of *inlG*, *inlH* and *inlE* was again determined by the semi-quantitative RT-PCR assay (Altrock, 1997; Bubert *et al.*, 1999) using RNA from BHI grown *L. monocytogenes* wild type and $\Delta inlG$, $\Delta inlH$ and $\Delta inlE$ mutant strains, with the *sod* gene as internal control. Again, RNA samples were digested with RNase-free DNase to avoid DNA contaminations and the complete digestion was verified by PCR as shown in Fig. 2.33. Isolated RNAs were reverse transcribed using the moloney murine leukemia virus reverse transcriptase and 10 μg total RNA as template. Diluted cDNAs were tested by PCR to determine optimal dilution for each gene (Fig. 2.34). For subsequent PCR amplifications, cDNAs were used in the dilutions 1:16 for the genes *inlG*, *inlH* and *inlE* and 1:1024 for *sod*.
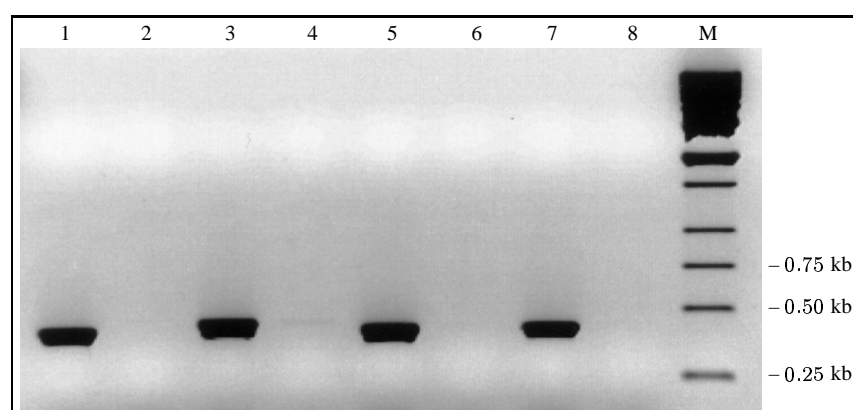
Figure 2.33: PCR analysis using RNA isolated from the *L. monocytogenes* wild type (lane 2) and the mutant strains $\Delta inlG$ (4), $\Delta inlH$ (6) and $\Delta inlE$ (8) after digestion of DNA with DNase prior to reverse transcription. PCRs were performed using the primer pairs RTG1up/RTG7Bdown or RTH4Bup/RTH6down (for $\Delta inlG$). None of the RNA samples yielded a PCR product, indicating that DNA was completely digested. As positive control, chromosomal DNA from the wild type strain was used (lanes 1, 3, 5 and 7).

2.14.1 Deletion of single genes from the *inlGHE* gene cluster did not alter transcription of the remaining *inl* genes

Transcription of the *inlGHE* gene cluster was examined in detail. As shown in Fig. 2.35, RT-PCRs using wild type RNA and primers derived from the coding region of *inlG*, *inlH*
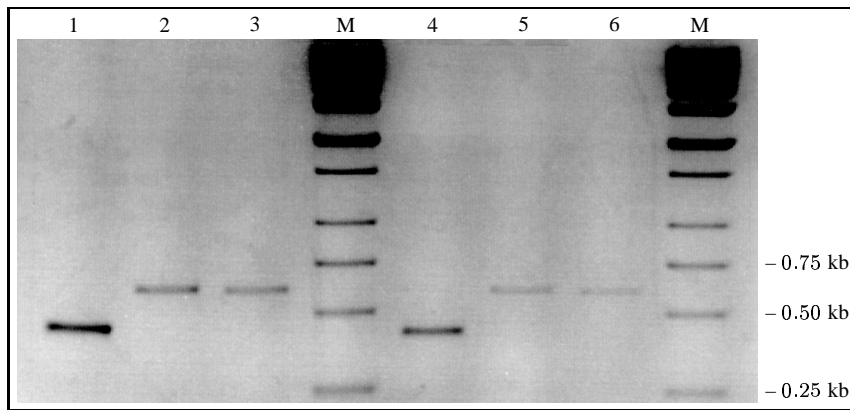
Figure 2.34: PCRs using primers derived from *inlG* (lanes 1 and 4), *inlH* (2 and 5) and *inlE* (3 and 6) to test for optimal dilutions of cDNAs. RNA was isolated from *L. monocytogenes* wild type at optical density of 180 Klett units and reverse transcribed as described above. The cDNAs used for PCRs were diluted 1:8 (1-3) and 1:32 (4-6).

and *inlE* yielded products of the expected sizes, demonstrating that all three genes are expressed, albeit at low efficiency compared to *sod*. Reactions with primers derived from *inlG* yielded higher amounts of product, confirming previous data which showed that transcription of this gene is stronger than that of *inlH* or *inlE* (see Fig. 2.9A). Transcription of the genes of the *inlGHE* cluster was next examined in the single mutants Δ*inlG*, Δ*inlH* and Δ*inlE*. The RT-PCRs shown in Fig. 2.35 demonstrated that the *inlG* gene was transcribed in the mutants Δ*inlH* and Δ*inlE* as efficiently as in the wild type strain, showing that deletion of *inlH* or *inlE* does not influence transcription of *inlG*. Similarly, transcription of *inlH* in the strains Δ*inlG* and Δ*inlE*, and of *inlE* in the strains Δ*inlG* and Δ*inlH* yielded the same level of transcripts as in the wild type strain, demonstrating that no alteration in gene expression resulted from the deletion events.

Next, a putative polycistronic transcription of the *inlGHE* gene cluster was examined. As shown in Fig. 2.36, basically no product was obtained by RT-PCRs using different primer pairs which should detect possible *inlG-inlH*, *inlH-inlE* or *inlG-inlH-inlE* polycistronic transcripts, except a faint but reproducible band obtained with primers to detect an *inlG-inlH* bicistronic transcript. Thus, these data suggest that the *inlG*, *inlH* and *inlE* genes are transcribed mainly as monocistronic transcripts. This is supported by the occurrence of promoter- and terminator-like structures up- and downstream of each of these genes which argue for their monocistronic transcription. However, the possibility that these genes are transcribed polycistronically at levels below those detectable by the applied RT-PCR method cannot be excluded.
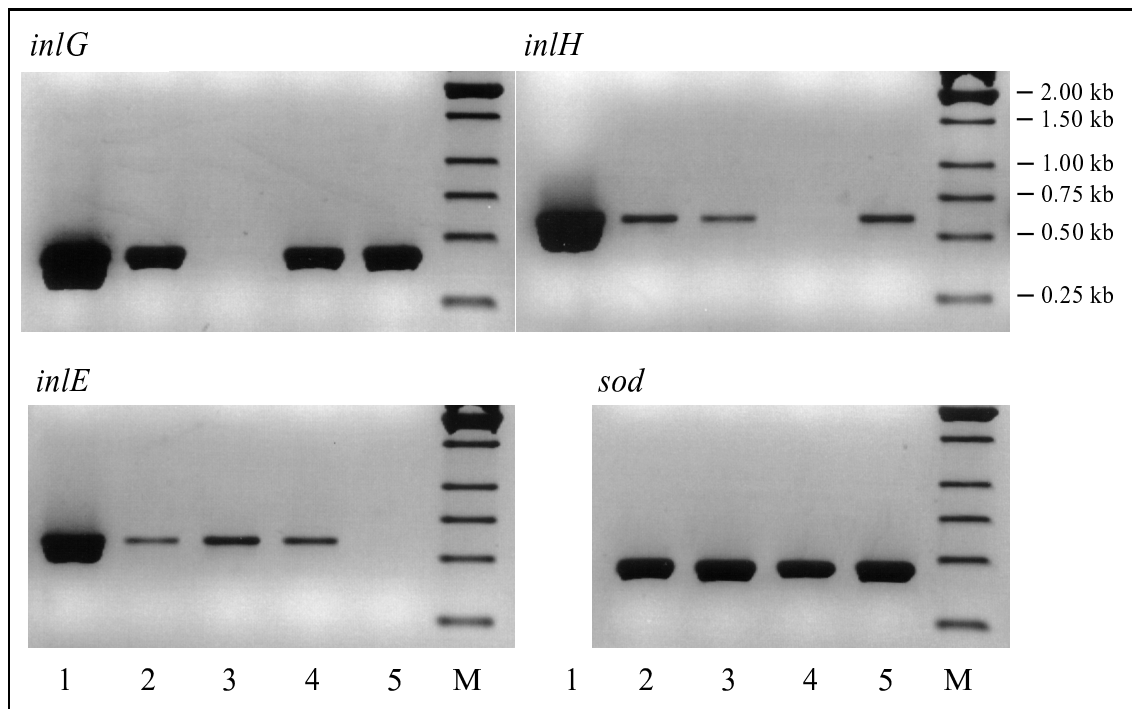
Figure 2.35: RT-PCRs. Transcription of the genes *inlG*, *inlH*, *inlE* and *sod* in the *L. monocytogenes* strains wild type (lane 2), $\Delta inlG$ (3), $\Delta inlH$ (4) and $\Delta inlE$ (5) in the logarithmic phase. The cDNAs were prepared from 10 μg total RNA and used in subsequent amplifications by PCR in the dilutions 1:16 for *inlG*, *inlH* and *inlE* and 1:1024 for *sod*. PCR products of *inlG* (432 bp), *inlH* (709 bp), *inlE* (606 bp) and *sod* (471 bp) were obtained with the primer pairs RTG1up/RTG7Bdown, RTH3up/RTH5down, RTE3Aup/RTE3Ddown and SOD-N/SOD-C, respectively. The DNA fragments were amplified from the 5' end of the coding region of the corresponding genes. 30 from 100 μl total volume of each PCR were loaded on agarose gels. Lane 1: Control PCR with chromosomal DNA.
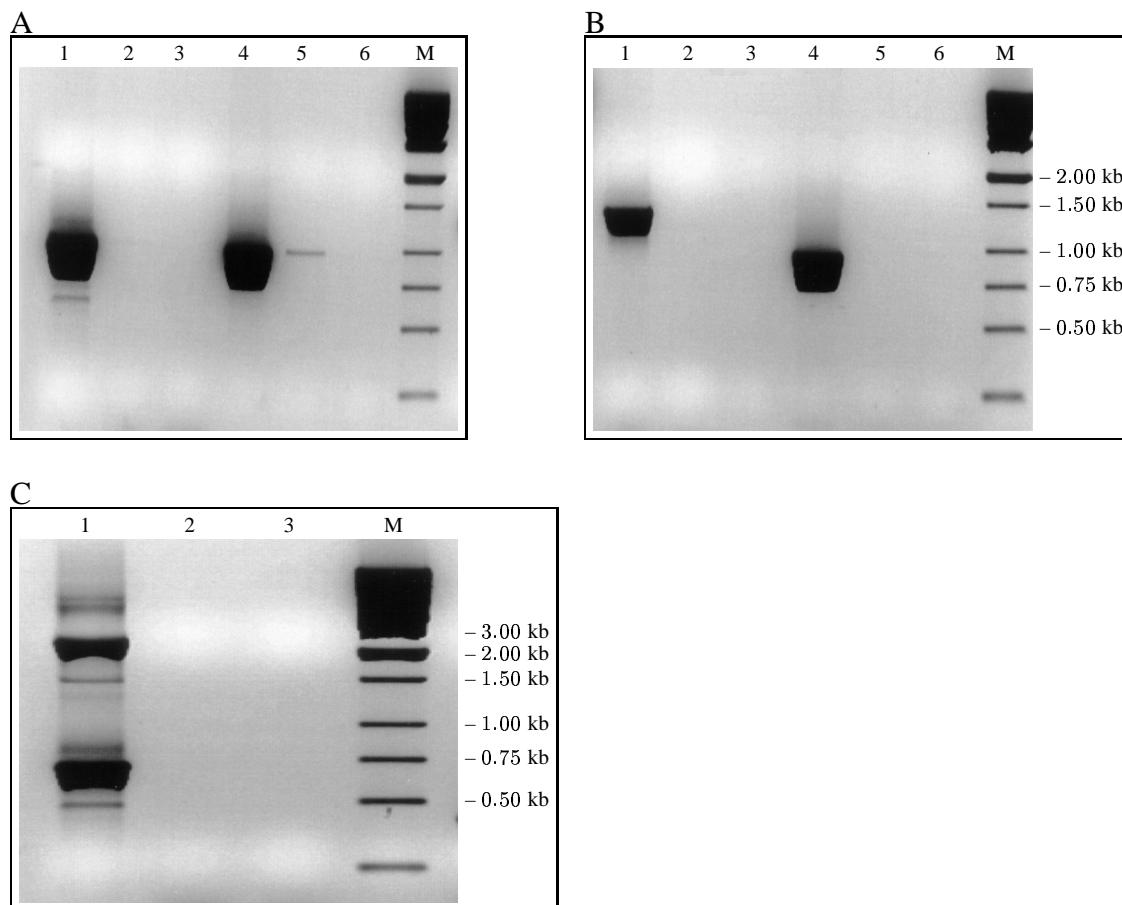
Figure 2.36: RT-PCRs to detect a polycistronic transcription of the *inlGHE* gene cluster in form from *inlG-inlH*, *inlH-inlE* and *inlG-inlH-inlE* transcripts using cDNA from the *L. monocytogenes* wild type strain in the dilutions 1:2 (lanes 2 and 5) and 1:4 (lane 3 and 6). The cDNAs were prepared from 10 μg total RNA. PCRs of *inlG-inlH* (971 bp), *inlH-inlE* (932 bp) and *inlG-inlH-inlE* (2,298 bp) were performed with the primer pairs inlX-2/RTH5down, inlYseq-1/inlZseq-3 and Xvorw-6/PinlZ, respectively. 30 from 100 μl total volume of each PCR were loaded on agarose gels. Lane 1: Control PCR with chromosomal DNA.

2.15 Transcription of the genes *inlG*, *inlH* and *inlE* is not activated by HepG-2 cells

Transcription of the genes *inlG*, *inlH* and *inlE* is very weak when *L. monocytogenes* grows extracellularly in rich culture media (BHI). Therefore, it was examined whether expression of these genes is induced after contact of the bacteria with mammalian cells. For this assay *L. monocytogenes* strains wild type and ΔinlA/B mutant containing the GFP reporter plasmids P*inlG-gfp*, P*inlH-gfp* and P*inlE-gfp* were used. Plasmids were introduced into *L. monocytogenes* by electroporation. Clones were selected by tetracycline resistance and screened by PCR as shown in Fig. 2.37.
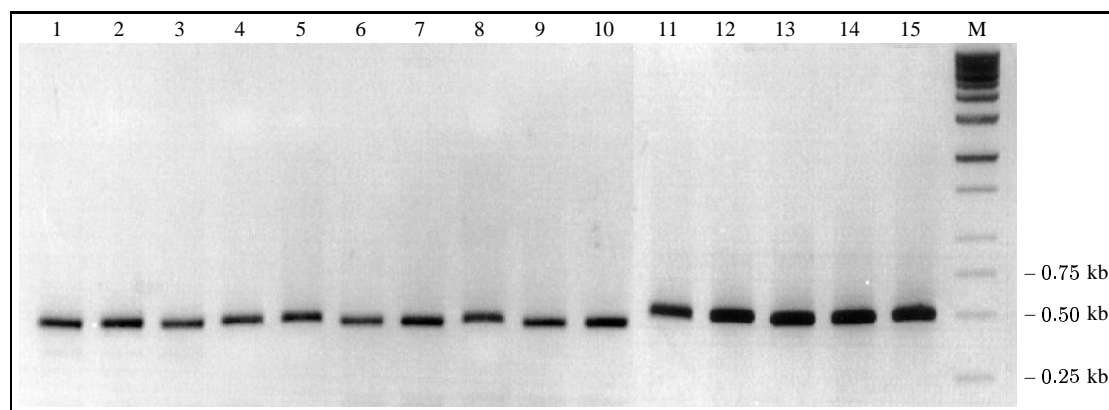
Figure 2.37: PCR analysis of clones of the *L. monocytogenes* ΔinlA/B strain containing the GFP expression plasmids P*inlG-gfp* (lanes 1-5), P*inlH-gfp* (6-10) and P*inlE-gfp* (11-15). PCR screening was performed with the primer pairs delseq-1/gfp230, inlX-2/gfp230 and delE-1/gfp230.

As described in detail in section 2.7, the plasmids P*inlG-gfp*, P*inlH-gfp* and P*inlE-gfp* carry the putative promoter regions of either *inlG*, *inlH* or *inlE* fused to the promoterless *gfp* cDNA encoding the green fluorescent protein GFP. The promoter activity of each of these genes measured as the GFP-mediated fluorescence was monitored by fluorescence microscopy while the bacteria were incubated in the presence of the human hepatocyte-like HepG-2 cells. Internalization of bacteria by these cells can be mediated by both internalins InlA and InlB. Bacteria were prepared for infection and applied to the cells as described in detail in section 5.4.4. As shown in Fig. 2.38 for *L. monocytogenes* strains carrying the plasmid P*inlH-gfp*, virtually no fluorescence was detected during an incubation period of 5 h post-infection, neither in the ΔinlA/B mutant, which was seen extracellularly adhered to the cells, nor in the wild type strain. Similar results were obtained with strains containing the two other plasmids P*inlG-gfp* and P*inlE-gfp* (data not shown). These results indicate that expression of *inlG*, *inlH* and *inlE* is not activated by contact or internalization of the bacteria into mammalian host cells of the cell line HepG-2. Similar results were obtained by FACS-analysis of Caco-2 cells infected with the *L. monocytogenes* wild type strain carrying the same GFP expression plasmids used here (Fig. 2.9B).
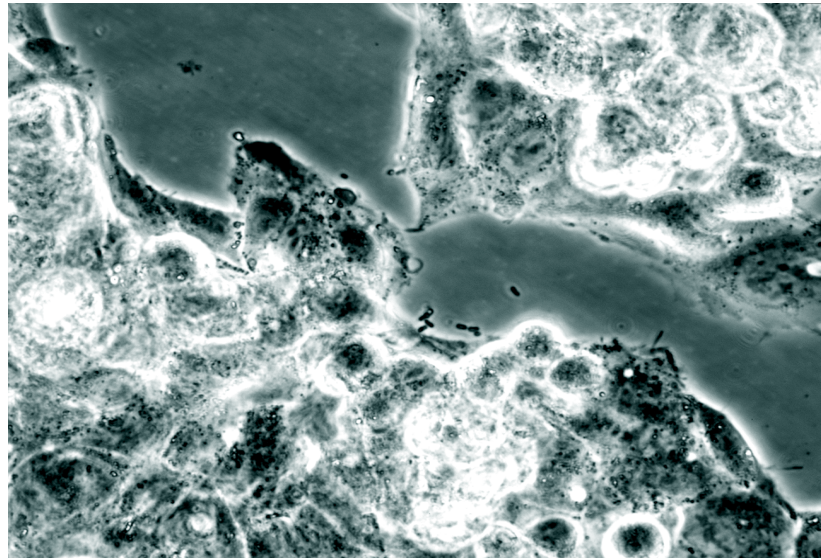
L. monocytogenes wild type x PinlH-gfp
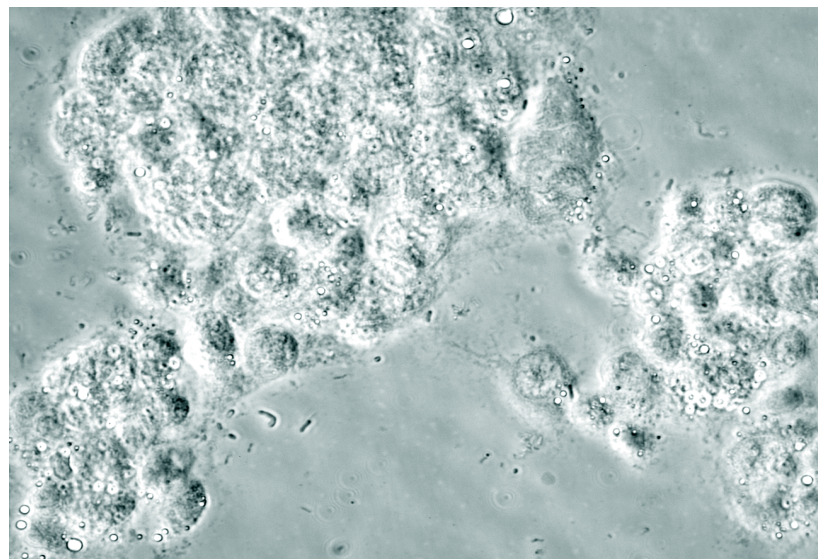
L. monocytogenes   inlAB x PinlH-gfp

Figure 2.38: Adhesion and invasion of *L. monocytogenes* strains harboring the GFP reporter plasmid P*inlH-gfp* into HepG-2 cells. No fluorescence could be detected in an incubation period of 5 h post-infection (Fluorescence pictures not shown). Photographs show phase-contrast microscopy images of HepG-2 monolayers containing bacteria of the strains wild type (upper panel) and $\Delta inlA/B$ (lower panel) transformed with the plasmid P*inlH-gfp*. Similar results were obtained with strains containing the other GFP expression plasmids P*inlG-gfp* and P*inlE-gfp*.

2.16   Identification of the *inlF* gene of *L. monocytogenes* EGD

In a previous study (Dramsi *et al.*, 1997), a new *inl* gene was identified in *L. monocytogenes* in addition to the *inlAB*, *inlC2DE* and *inlGHE* gene clusters. This gene, termed *inlF*, encodes a large internalin of 821 amino acids similar to InlA. Nucleotide sequencing of the chromosomal region surrounding *inlF* revealed that this gene is not clustered with further internalin genes. Upstream of *inlF* there exists an ORF with no similarities to sequences in the databases, while downstream of *inlF* an ORF was detected which encodes a protein similar to the phosphoenolpyruvate synthase of *E. coli*. Similarly to the other large internalin genes, *inlF* is maximally expressed during the early exponential growth phase under extracellular conditions. Deletion of *inlF* did not affect entry of *L. monocytogenes* into non-phagocytic cells *in vitro*. Also, virulence of the *inlF* deletion mutant was not altered in the mouse model (Dramsi *et al.*, 1997).

Since the gene cluster *inlC2DE* identified previously (Dramsi *et al.*, 1997) was not detected in other *L. monocytogenes* strains (see Fig. 2.3), the occurrence of the *inlF* gene in other strains than that used in Dramsi *et al.* (1997) was tested in the present study. Oligonucleotides derived from the published *inlF* sequence were used to amplify the complete *inlF* gene by PCR from *L. monocytogenes* strains EGD, NCTC 7973 and LO28. None of the reactions performed with *inlF*-specific primers yielded products, but control PCRs with the *L. monocytogenes*-specific primers MonoA and MonoB resulted in bands of the expected sizes. To investigate whether the *inlF* gene is absent in the strains tested, further internal primers were designed that allowed amplification of *inlF* in two overlapping fragments, termed inlF1 and inlF2. Again inlF1 could not be amplified, while the second PCR gave a product, which was however smaller than expected. The PCR product inlF2 from strain EGD, which was obtained with the primers inlF-2 and inlF-6, was cloned into the vector pUC18 and sequenced. The nucleotide sequence of this DNA fragment revealed a high identity to the existing *inlF* sequence. To extend the sequence from the strain EGD, inverse PCRs were performed in both up- and downstream directions as previously described (Raffelsbauer, 1997) using *Alu*I-, *Dra*I- and *Rsa*I-cleaved chromosomal DNA and the primer pair inlFinv-1/inlFinv-2. The PCR products obtained were cloned into pUC18 and sequenced. By this procedure, a chromosomal region of 1,201 bp was amplified which contained an ORF encoding an internalin-like protein. As shown in Fig. 2.39, the sequence of the DNA region amplified was nearly identical to the previously determined *inlF* sequence (Dramsi *et al.*, 1997), showing that this gene is also present in strain EGD. The complete nucleotide sequence of the fragment of the *inlF* gene from *L. monocytogenes* EGD amplified in this study is shown in App. B.3.

2.17   The *inlGHE* gene cluster in *L. monocytogenes* clinical isolates

The *L. monocytogenes* EGD strain used in this study, provided by S. H. E. Kaufmann (University of Ulm, Germany), belongs to the serotype 1/2a. This strain causes sep-

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        BESTFIT of: inlF (EGD)  check: 5078  from: 1  to: 1201
         to: inlF.seq  check: 1679  from: 1  to: 2466
            Gap Weight:      50      Average Match: 10.000
            Length Weight:    3      Average Mismatch: -9.000

             Quality:  11953          Length:     1201
             Ratio:    9.953          Gaps:          0
        Percent Similarity: 99.750   Percent Identity: 99.750

            Match display thresholds for the alignment(s):
                            | = IDENTITY


                 .         .         .         .         .
      1 AGCTGGCTTGACAAAATTGGACAATATAGCAGCATATTCGAATAAAATCA 50
        |||||||||||||||||||||||||||||||||||||||||||||||||
    684 AGCTGGCTTGACAAAATTGGACAATATAGCAGCATATTCGAATAAAATCA 733

                 .         .         .         .         .
     51 CTGATATTACTCCTGTGACCAATTTAACAAGACTCCAGTATTTGGATTTA 100
        |||||||||||||||||||||||||||||||||||||||||||||||||
    734 CTGATATTACTCCTGTGACCAATTTAACAAGACTCCAGTATTTGGATTTA 783

                 .         .         .         .         .
    101 GGTAGTAATGAAATCACTGATTTAAGTCCTGTGGCTAATCTGCAAAAATT 150
        |||||||||||||||||||||||||||||||||||||||||||||||||
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                 .         .         .         .         .
    201 AAGATTTAACAAATTTAACTTCGTTGGGTTTACAAAACAATAAAATTAGT 250
        |||||||||||||||||||||||||||||||||||||||||||||||||
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                 .         .         .         .         .
    251 GATATATCCGTTTTGAAAAATCTAACCCATGTGACTTATTTGCAGCTGGG 300
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                 .         .         .         .         .
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        |||||||||||||||||||||||||||||||||||||||||||||||||
   1034 CAAGTTTGCAGTTAACACAAAACCATATTACTGACATAAGTCCTTTAGCC 1083

                 .         .         .         .         .
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        |||||||||||||||||||||||||||||||||||||||||||||||||
   1084 AACTTAACCAAAATACAATACTCTGACTTCTCTAATCAGATGATAACAAA 1133


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    801 ATGGTTGGTACGACGCAGAAACAGGCGGAACAAAATGGGACTTCACAACC 850
        |||||||||||||||||||||||||||||||||||||||||||||||||
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                 .         .         .         .         .
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   1151 AATGAAACGATAGCATACGATACCTTACTCAATGAACCGACCACTCCAAC 1200
        |||||||||||||||||||||||||||||||||||||||||||||||||
   1834 AATGAAACGATAGCATACGATACCTTACTCAATGAACCGACCACTCCAAC 1883
```

Figure 2.39: Nucleotide sequence comparison of the *inlF* gene amplified from the EGD strain used in this study (upper line) and the by Dramsi *et al.* (1997) determined *inlF* sequence (under line) created by the program BestFit (Genetics Computer Group, University of Wisconsin). Pipes indicate identical nucleotides. Nucleotide positions within the sequences are listed at both margins.

ticaemia and infections of the central nervous system like meningitis and meningoencephalitis. Other *L. monocytogenes* strains were however described which can cause a localized disease in form of gastroenteritis. Three of these strains were used in the present study to investigate the occurrence of Inl proteins and putative variations in the internalin equipment in comparison to the strain EGD. The following *L. monocytogenes* gastroenteritis isolates were used: Strain 1724, a clinical isolate from Denmark (1995), belongs to serotype 4b (Heitmann *et al.*, 1997). Strain 35264, also a clinical isolate (Germany), was ascribed to the serotype 1/2a (H. Hof, University of Mannheim, Germany). Strain 1727 was isolated from milk in the USA (1995) and belongs to the serotype 1/2b (Dalton *et al.*, 1997). In addition, three other sepsis causing *L. monocytogenes* isolates were used. Strain 579 was isolated in the USA (1985) from cheese (Linnan *et al.*, 1988). Strains 535 and 536 are clinical isolates from the blood of patients from Switzerland (1983-1987) (Bille, 1998). All three sepsis strains belong to the serotype 4b.

In the present study the question was raised whether the *L. monocytogenes* gastroenteritis strains are impaired to spread through the organism due to the lack of some internalins, resulting in a form of listeriosis which is restricted to the region of the entry portal, the gastrointestinal tract. Therefore, the occurrence of the *inlGHE* gene cluster in the gastroenteritis strains compared to the sepsis isolates described above was examined by PCR. As control, strain EGD was used.

As shown in Fig. 2.40, PCRs using oligonucleotides that specifically detect the occurrence of the *inlG* gene unequivocally showed that this gene is present in the gastroenteritis strains 1724, 35264 and 1727. Strain 35264 yielded two- to four-fold higher amounts of PCR product than the control strain EGD. Faint DNA bands were also detected in the sepsis strains 535 (A1) and 579 (A2), whereas strain 536 was negative in both PCR assays. PCRs with *inlH* specific primers showed divergent results. As depicted in Fig. 2.40, in the first assay (B1) the *inlH* gene could be detected only in the gastroenteritis strain 35264. In contrast, the same PCR yielded in the remaining strains several bands of lower intensities. These results were confirmed by a second PCR performed with another primer pair (data not shown). However, a third assay (B2) revealed the presence of *inlH* in the strains 1724, 35264 and 535. In all three assays the control strain EGD yielded specific products. In accordance with the data obtained with *inlG* and *inlH* specific primers, the *inlE* gene was detected in the strains 1724, 35264 and 535. As shown in Fig. 2.40C, faint DNA bands were also obtained with the remaining strains 579, 1727 and 536. The control PCR with *L. monocytogenes* specific primers derived from the *iap* gene yielded in all strains tested products in the expected size (2.40D). The above data demonstrate that at least two of the three gastroenteritis strains tested show to possess the *inlGHE* gene cluster. However, the genes *inlG*, *inlH* or *inlE* could not be clearly detected in the sepsis strains 579 and 536. Taken together, these results give evidence of variations within the sequences of the genes from the *inlGHE* gene cluster among the *L. monocytogenes* isolates tested and suggest the occurrence of different equipments of internalins in these strains.
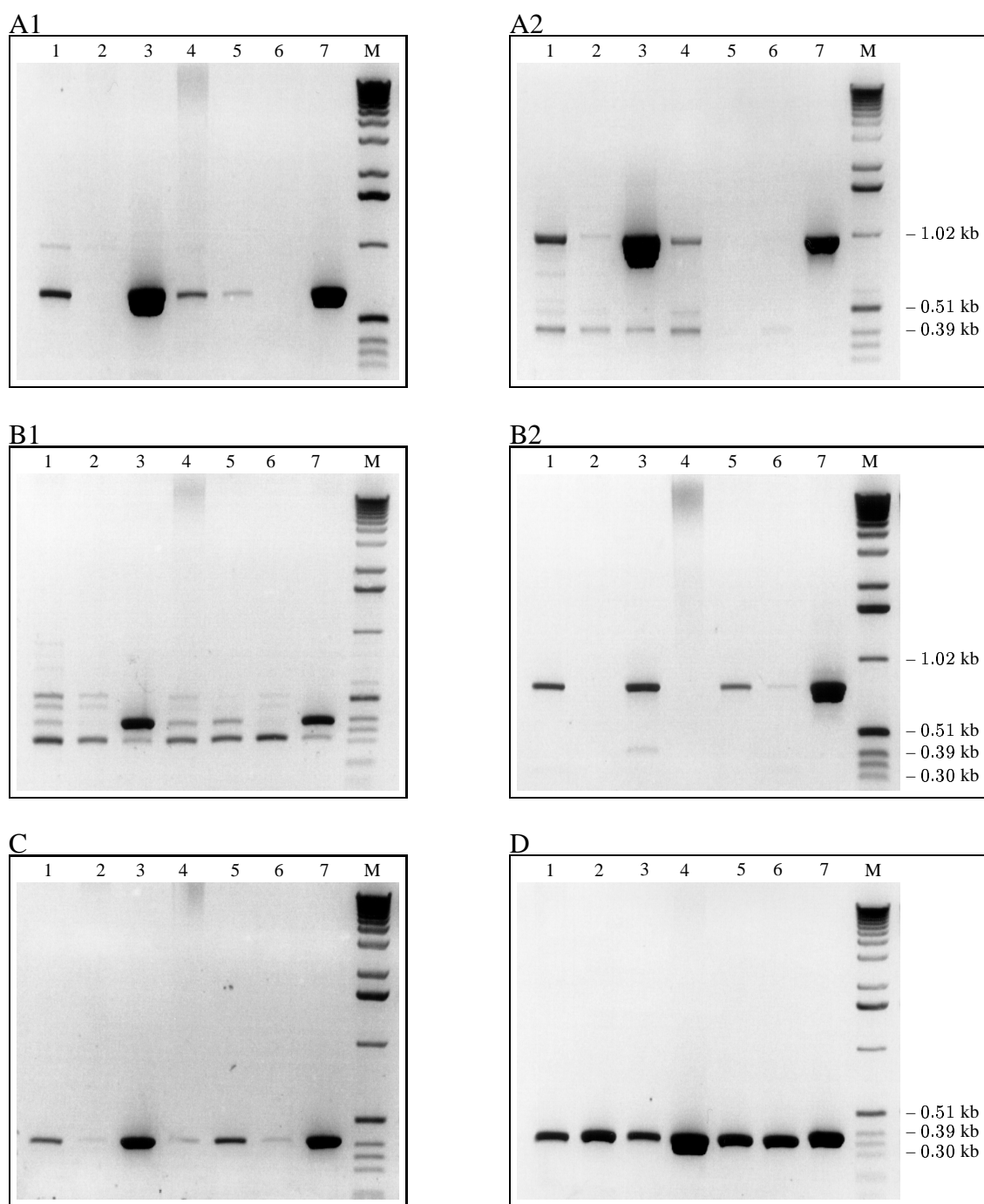
Figure 2.40: PCRs to detect the genes *inlG* (A1 and A2), *inlH* (B1 and B2), *inlE* (C) and *iap* (D) in the *L. monocytogenes* isolates 1724 (1), 579 (2), 35264 (3), 1727 (4), 535 (5), 536 (6) and EGD (7). Strains 1, 3 and 4 cause gastroenteritis, whereas strains 2, 5 and 6 cause sepsis. Strain EGD (7) was used as control. PCRs were performed with following primer pairs. Length of the obtained PCR product is indicated. A1. pGluco-1/inlX-3 (*inlG*; 664 bp). A2. pGluco-1/inlXspez-1 (*inlG*; 1,001 bp). B1. inlX-2/inlYC2spez-1 (*inlH*; 397 bp). B2. inlYC2spez-2/PinlZd (*inlH*; 794 bp). C. lism51-26/ORFZ-1 (*inlE*; 412 bp). D. MonoA/MonoB (*iap*, 370 bp).

2.18   *L. monocytogenes* clinical isolates and cell death

The ability of the *L. monocytogenes* clinical isolates described above to invade and grow in mammalian cells *in vitro* was examined in detail somewhere else (Wagner *et al.*, manuscript in preparation). This study revealed differences in adhesion, invasiveness and intracellular replication in the cell lines Caco-2, TIB73, J774 and P388 among the strains tested, which could at least partially explain the distinct behaviors of these strains during a human listeriosis. In the present study, the fate of cells infected with these *L. monocytogenes* strains was investigated. In particular, the aspects cell viability and cell death were examined.

Induction of cell death (necrosis or apoptosis) by infection with *L. monocytogenes* has been previously observed in lymphocytes, dendritic cells and hepatocyte-like cells (Merrick *et al.*, 1997; Barsig and Kaufmann, 1997; Guzmán *et al.*, 1996; Rogers *et al.*, 1996). The present study investigated whether the distinct *L. monocytogenes* clinical isolates described above affect cell viability by inducing apoptosis or necrosis in cells of the epithelial cell line Caco-2 and the macrophage-like cell line J774. The *L. monocytogenes* isolates used here differ from each other by causing a localized (gastroenteritis) or a generalized (septicaemia) form of listeriosis *in vivo*. Induction of cell death with release of the bacteria to the extracellular environment could impair bacterial replication and propagation. In addition, cell death by necrosis but not by apoptosis elicits an inflammatory response.

Necrosis and apoptosis have distinct morphological and biochemical hallmarks. One biochemical feature that characterizes cell death by apoptosis is the activation of an endogenous endonuclease that cleaves genomic DNA at the region between nucleosomes (Wyllie, 1980; Duke, 1983), thus generating mono- and oligonucleosomal DNA fragments which can be detected in the cytoplasm of dying cells hours before membrane breakdown. Here, induction of apoptosis due to infection with *L. monocytogenes* was studied by photometrically determining the amounts of histone-associated DNA fragments present in the cytoplasm of infected Caco-2 and J774 cells using the Cell Death Detection ELISA^{PLUS} Kit (Roche) as described in detail in section 5.4.5. Furthermore, evidences of cell disintegration caused either by necrosis or as a later event of the apoptotic process (secondary necrosis) were obtained by simultaneous analysis of the culture supernatant. These experiments were performed together with Dr. M. Wagner at the University of Würzburg, Germany.

As summarized in Fig. 2.41, no significant differences in the amount of histone-associated DNA fragments were observed between strain EGD and non-infected cells (NC), suggesting that infection of Caco-2 and J774 cells with this strain does not elicit apoptosis. However, considerable differences were detected among the distinct *L. monocytogenes* isolates concerning their ability to induce cell death in comparison to strain EGD. As shown in Fig. 2.41A, a two- to three-fold increase in the level of histone-associated DNA fragments in comparison to strain EGD was detected in the cytosol of Caco-2 cells infected with the strains 535 and 1724, suggesting that these isolates induce apoptosis in this epithelial cell line. The photometric determination of the DNA fragments in these samples yielded optical densities of nearly 2.0, which are similar to the values obtained when the apoptosis-inducing substance campothecin was used in control

experiments. In contrast, very few nucleosomes could be found in the culture supernatant (data not shown), indicating that no significant changes in cell membrane integrity were measurable at lest at the time point of analysis. High amounts of DNA fragments (1.6-fold increase in comparison to EGD) were also detected in the cytoplasm of cells infected with strain 1727, whereas strain 579 apparently did not have any effect on cell viability. Infection of Caco-2 cells with the $\Delta inlGHE$ mutant led to a two-fold increase in DNA fragmentation, which correlates to the increased invasiveness of the mutant into this cell line. In contrast, infection with the $\Delta inlC$ mutant shows a reduced amount of nucleosomes (45% compared to strain EGD). A correlation between the amount of DNA fragments detected in the present study and the bacterial invasiveness (Wagner *et al*., manuscript in preparation) was also observed for the *L. monocytogenes* clinical isolates tested. Interestingly, the wild type strain EGD failed to elicit an increased DNA fragmentation under the conditions used in comparison to non-infected cells. This result is in accordance with microscopical studies using the dyes Hoechst 33342 and propidium iodide which showed that infection of Caco-2 cells with strain EGD led to an intensive spreading of the bacteria without signs of significant cell death, in contrast to strain 1724, which caused a pronounced cell destruction (Wagner *et al*., manuscript in preparation). In J774 cells no significant alteration in the amount of DNA fragments was observed between strains EGD and $\Delta inlGHE$, which was only slight increased in comparison to the non-infected control cells. However, strains $\Delta inlC$, 1727, 1724, 579 and 535 show a growing increase in DNA fragmentation. In this case, no direct correlation between induction of cell death and bacterial invasiveness could be observed. However, strains with high levels of nucleosomes were shown to spread more efficiently in J774 cells.

A

B
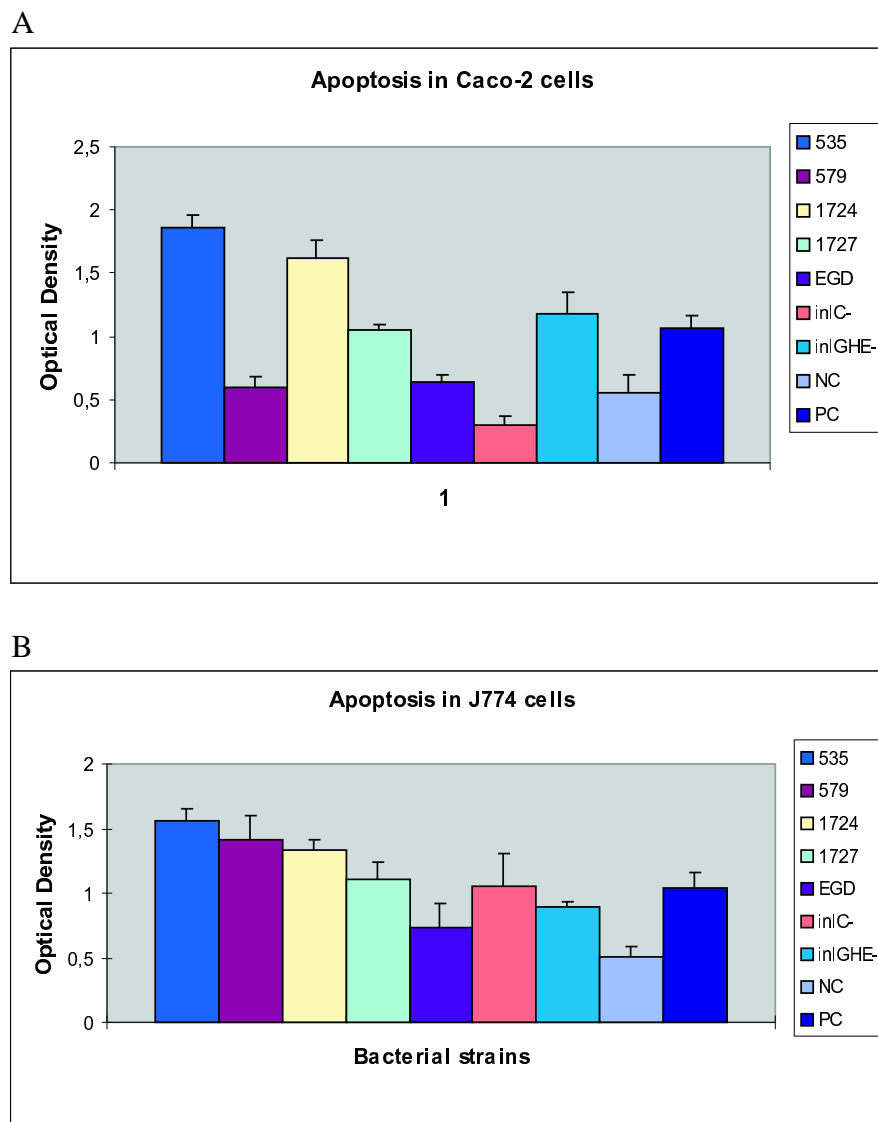
Figure 2.41: Analysis of cell death by apoptosis induced by infection with different *L. monocytogenes* isolates. Quantitative photometric determination of histone-associated DNA fragments after infection of Caco-2 (A) and J774 (B) cells with *L. monocytogenes* strains 535, 579, 1724, 1727, EGD, $\Delta inlC$ and $\Delta inlGHE$. NC and PC represent the negative control using non-infected cells and the positive control supplied by the kit, respectively. Cells were infected for 1 h at a MOI of 20 (Caco-2) or 0.5 (J774) bacteria per cell. After an incubation time of 9 h (Caco-2) or 6 h (J774), contents of histone-associated DNA fragments were determined photometrically using the enzyme-immunoassay Cell Death Detection ELISA^{PLUS} (Roche). Graph presents means $\pm$ standard deviations of four independent samples per strain from one representative experiment.

Discussion

In the present study three new genes of *Listeria monocytogenes* belonging to the internalin multigene family were identified and characterized (Raffelsbauer *et al.*, 1998). These genes, termed *inlG*, *inlH* and *inlE*, encode proteins of 490, 548 and 499 amino acids, respectively, which show all features characteristic for the large internalins. InlA and InlB were the first members of this family to be described (Gaillard *et al.*, 1991). Both are surface proteins required for invasion of *L. monocytogenes* into mammalian cells (reviewed by Kuhn and Goebel, 2000). InlA mediates entry into the enterocyte-like cell line Caco-2 (Gaillard *et al.*, 1991), whereas InlB triggers invasion into several other cell lines, including endothelial cells, hepatocytes and some epithelial cells lines (Greiffenberg *et al.*, 1998; Parida *et al.*, 1998; Ireton *et al.*, 1996; Dramsi *et al.*, 1995; Lingnau *et al.*, 1995). A third member of this family, termed InlC or IrpA, was identified later (Engelbrecht *et al.*, 1996; Domann *et al.*, 1997). InlC is a small extracellular protein of unknown function, but seems to be important for virulence of *L. monocytogenes*, since the absence of this protein leads to an increased LD_{50} in the mouse model (Engelbrecht *et al.*, 1996).

As reported in Raffelsbauer *et al.* (1998), the internalins InlG, InlH and InlE display all features characteristic for the large, cell wall-bound internalins, whose prototype is represented by InlA: a 33 to 35-amino acids signal sequence characteristic for Gram-positive bacteria showing a typical signal peptidase 1 cleavage site, a leucine-rich repeat (LRR) region or region A, a conserved inter-repeat region, a further repeat region containing the B repeats, and a putative cell wall anchor at the C-terminus which fixes the proteins on the bacterial cell surface.

The leucine-rich repeats, which are characteristic for the superfamily of leucine-rich repeat (LRR) proteins, are built by consecutive repeat units of regularly spaced leucine or isoleucine residues displaying a characteristic consensus sequence (Kobe and Deisenhofer, 1994; Kobe and Deisenhofer, 1995). LRRs correspond to β–α structural units consisting of a short β-strand and an opposing antiparallel α-helix connected to each other by coils (Marino *et al.*, 2000). These structures results in a non-globular, horseshoe-shaped molecule, where the β-strands form the concave and the α-helices the convex face of the molecule. The LRR region of internalins consists of varying numbers of LRR units of 22 amino acids each. The number of these units ranges from four in InlG and 14 in InlA (Raffelsbauer *et al.*, 1998). Interestingly, in the internalins InlB, InlC2, InlD, InlE, InlG and InlH the LRRs number 5 and 9 were fused to each other, leading to the complete deletion of the repeats 6, 7 and 8, and reducing the number of LRRs in units of four. This deletion was only partially observed in the larger internalin InlF (821 amino acids). In addition, repeats 2, 3 and 11 were absent in InlG (4 LRRs), but present in InlH and InlE (both 7 LRRs), whereas repeats number 12, 13 and 14 are missing in all three internalins. Such internal deletion of LRR units may arise by homologous recombination between the

highly similar nucleotide sequences encoding these repeats. These variations in the number of repeats by intragenic recombination may generate new proteins. A similar example of internal deletion of LRRs was also observed in i-InlE, a small internalin of *L. ivanovii* which has only two repeats in contrast to the remaining small internalins with five LRRs (Engelbrecht *et al.*, 1998b). Therefore, it is tempting to speculate that the internalin multigene family arised by gene duplication followed by homologous recombination, leading to deletions and variations in the structure of these highly similar proteins. A comparable mechanism was previously suggested for the evolution of eukaryotic LRR proteins, which comprises unequal cross-overs and duplications of gene fragments corresponding to prototypic leucine-rich building blocks (Kobe and Deisenhofer, 1994). Interestingly, InlA is mostly conserved in a wide variety of *L. monocytogenes* strains (Poyart *et al.*, 1996). It was shown that the region A of InlA derived from 68 *L. monocytogenes* wild type strains displays a DNA polymorphism which arises exclusively from point mutations, indicating that the genetic heterogeneity exhibited by these repeats is not caused by intragenic recombinations. The conservation of InlA, which points to the importance of this protein due to its function, and the intragenic recombination between LRRs observed in other Inl proteins suggest that InlA may be a prototype from which the internalin multigene family arises.

LRR proteins are mostly involved in protein-protein interactions and act either as adhesive proteins, like the small proteoglycans biglycan, fibromodulin and decorin, or as receptors in signal transduction cascades like CD14 and gonadotropin receptors (Kobe and Deisenhofer, 1994; Kobe and Deisenhofer, 1995). These protein binding functions seem to be performed by the LRR structures. Indeed, it was recently shown that the LRR regions of InlA and InlB are essential for interactions with mammalian cells by binding to the corresponding receptors (Lecuit *et al.*, 1997; Braun *et al.*, 1999). The LRR region of InlB, whose x-ray crystal structure was recently determined (Marino *et al.*, 1999), is necessary and sufficient for the activation of phosphoinositide 3-kinase, rearrangement of the actin cytoskeleton and invasion of permissive cells *in vitro* (Braun *et al.*, 1999). Similarly, the LRR region of InlA is necessary to mediate invasion in mammalian cell *in vitro* but requires the presence of the inter-repeat region for this activity (Lecuit *et al.*, 1997). The specificity of these interactions was demonstrated with E-cadherin, the cellular receptor of InlA. Substitution of one single amino acid at position 16 (which is proline in the human E-cadherin) abrogates interaction (Lecuit *et al.*, 1999). Therefore, it will be interesting to study the function of the smaller LRR regions of the newly identified InlG, InlH and InlE and elucidate whether and how variations in sequence and size of the LRR regions influence the ability of internalins to interact with mammalian cells.

Whereas the role of the LRR regions of InlA and InlB is well characterized, very little is known about region B. InlA has three B repeats of 70, 70 and 49 amino acids, respectively (Gaillard *et al.*, 1991). In InlG and InlH the first B repeat is missing. Surprisingly, compared to all other large internalins, InlE possesses a smaller region B with only one repeat of 47 amino acids, and additionally a third region of two repeats composed of 20 amino acids each, designated as region D, which is similar to the B repeats (Raffelsbauer *et al.*, 1998). This may be another example of intragenic recombination between homologous repeats of the internalins. Interestingly, InlB has also only one B repeat consisted of 56 amino acids, and in addition a third repeat region termed region C (Dramsi *et al.*,

1997). Whereas the function of the B and D repeats remains unknown, it was recently shown that the region C is involved in the anchorage of InlB to the bacterial surface (Braun *et al.*, 1997; Jonquieres *et al.*, 1999). It is interesting to note that, whenever a third repeat region (region C of InlB or region D of InlE) is present, region B is shorter and consists of only one repeat. It is also intriguing that the similarity of InlE is higher to InlB than to InlA (in contrast to InlG and InlH), but InlE is anchored to the bacterial surface similarly to InlA via a LPXTG motif and not by a repeat region as InlB (Raffelsbauer *et al.*, 1998; Braun *et al.*, 1997).

Except InlC, *L. monocytogenes* internalins are surface-bound proteins. The internalin cell wall anchor consists of a pentapeptide with the consensus sequence LPXTG, which is followed by a region of about 20 hydrophobic amino acids spanning the cell membrane and a short tail of positively charged residues which act as stop transfer signal (Gaillard *et al.*, 1991; Lebrun *et al.*, 1996). This signature is characteristic for surface proteins of Gram-positive cocci such as protein A of *Staphylococcus aureus* and M-protein of *Streptococcus pyogenes* (reviewed by Navarre and Schneewind, 1999). Anchoring seems to occur by a covalent linkage between the carboxyl group of the threonine from the LPXTG motif and a free amino group of the peptidoglycan (Schneewind *et al.*, 1995; Navarre and Schneewind, 1994). Sortase, the enzyme responsible for surface protein anchoring, was recently purified from *S. aureus* and its activity and mechanism characterized (Ton-That *et al.*, 1999; Mazmanian *et al.*, 1999). Sortase is a transpeptidase which cleaves the protein between threonine and glycine of the LPXTG motif and links the N-terminal part of the protein to the peptidoglycan of the cell wall by an amide linkage between the carboxyl group of threonine and a free aminogroup of one of the glycines from the pentaglycine crossbridge of *S. aureus* (Schneewind *et al.*, 1995; Mazmanian *et al.*, 1999). In case of InlA of *L. monocytogenes*, linkage is established between the carboxyl group of threonine and a free amino group of the meso-diaminopimelic acid crossbridge of the peptidoglycan (Dhar *et al.*, 2000).

Similarly to InlA, all new three internalins InlG, InlH and InlE possess the cell wall anchor LPXTG motif at the C-terminal part of the molecule, which probably attaches these proteins to the bacterial cell surface (Raffelsbauer *et al.*, 1998). Interestingly, InlE is the only one of these internalins whose pentapeptide LPITG corresponds to the consensus motif. In InlG and InlH the corresponding pentapeptides LPKTS and LPTAG differ from the consensus sequence by the exchange of glycine by serine at position 5 and threonine by alanine at position 4, respectively. Since antibodies against these proteins are not available, it could not be verified whether these substitutions affect protein association at the cell surface. However, it is worthwhile noting that both InlC2 and InlD have the same pentapeptide as InlH (Raffelsbauer *et al.*, 1998; Dramsi *et al.*, 1997).

Small amounts of InlA were detected in the supernatant, but this extracellular fraction may be derived from proteolytic cleavage of surface-bound InlA. In contrast, InlB is found in the supernatant in much larger amounts, which indicates a looser anchorage of this protein to the bacterial surface (Dramsi *et al.*, 1993; Lingnau *et al.*, 1995). Indeed, InlB exists in secreted and surface-bound forms. Both forms can trigger signaling in the host cells, but eliciting different events. While surface-bound InlB triggers internalization of *L. monocytogenes* without causing significant morphological changes in the cell surface, soluble InlB promotes membrane ruffling of the cell, a process observed in the trigger

mechanism of entry commonly used by *Salmonella* and *Shigella* (Braun *et al*., 1999; Ireton *et al*., 1999; Braun *et al*., 1998; Swanson and Baer, 1995). InlB is the only large internalin that displays a mechanism of protein association different from the LPXTG anchor motif. The protein is attached to the bacterial cell surface by a 232-amino acid region located at the C-terminal part of the molecule. This cell surface anchor region, previously called region C, is organized in 3 repeats of 80 amino acids beginning with the sequence GW, i. e. glycine-tryptophan (Braun *et al*., 1997; Jonquieres *et al*., 1999). These GW repeats or modules represent a novel anchor motif which is also present in Ami, a newly identified surface-associated bacteriolysin of *L. monocytogenes*, and in lysostaphin, a bacteriocin of *S. simulans* (Braun *et al*., 1997). Interestingly, when exogenously added to the medium, purified InlB can associate to the bacterial surface of *L. monocytogenes* and even of other Gram-positive bacteria such as *Bacillus* and *Staphylococcus* spp. (Braun *et al*., 1997). This association of InlB at the bacterial surface uses lipoteichoic acids of the cell membrane as ligands (Jonquieres *et al*., 1999).

The genes *inlG*, *inlH* and *inlE* are clustered together in the same orientation in a 5 kb long region of the chromosome and are flanked by two house keeping genes encoding proteins with homology to the 6-phospho-β-glucosidase and the succinyl-diaminopimelate desuccinylase of *E. coli* (Raffelsbauer *et al*., 1998). In the same position on the chromosome of another *L. monocytogenes* EGD strain a similar gene cluster was identified which also contains three *inl* genes, *inlC2*, *inlD* and *inlE* (Dramsi *et al*., 1997). The comparison of both gene clusters, *inlGHE* and *inlC2DE*, reveals some interesting new aspects. Firstly, the *inl* gene cluster identified in the present study begins with *inlG*, a novel gene encoding a large internalin which was apparently not recognized in the previous study. Secondly, there is another new *inl* gene in this gene cluster, termed *inlH*. This gene seems to be generated by homologous recombination between two identical repeat sequences in the B regions of *inlC2* and *inlD*, respectively. These two genes are located adjacent to each other in the previously described *inlC2DE* cluster. The newly generated *inlH* gene hence encodes a chimeric internalin consisting of the N-terminal part of InlC2 and the C-terminal part of InlD. The *inlG* gene is most likely also present in the *inlC2DE* gene cluster since the sequence deposited in the Gen Bank database shows the 3'-terminal end of *inlG*. Furthermore PCR analysis using chromosomal DNA derived from *L. monocytogenes* isolates of many of the known serotypes detected the presence of *inlG* and *inlH* in most strains tested (Raffelsbauer *et al*., 1998). In contrast, the *inlC2DE* cluster could not be detected in any of the strains tested, suggesting that this cluster might be specific for the EGD strain used in the previous study (Dramsi *et al*., 1997). In addition, the *inlC2* and *inlD* genes seem to be also absent in the recently sequenced *L. monocytogenes* strain EGDe, whereas the remaining internalin genes *inlA*, *inlB*, *inlE*, *inlF*, *inlG* and *inlH* are present (The European *Listeria* Genome Consortium).

The proteins InlC2, InlD, InlE, InlG and InlH show the most pronounced homology among the internalin family. The percent similarities vary from 98% between InlC2 and InlH to 58% between InlG and InlD. Among the proteins derived from the *inlGHE* gene cluster, similarity was highest between InlH and InlE (68%). This striking conservation was also observed previously at the DNA level (Raffelsbauer, 1997). The high identity between the genes of the *inlGHE* cluster, especially between *inlH* and *inlE*, suggests that this cluster may have arisen by gene duplication. High similarity rates were also detected

between the proteins InlC2, InlD and InlE (Dramsi *et al.*, 1997). In general, the proteins InlC2 to InlH seem to be more related to InlA than to InlB. Interestingly, InlB is the most divergent member of the internalin family and the small internalin InlC is more similar to InlB than to InlA.

Multigene families are very common in eukaryotes. However, few examples were found so far in prokaryotes. The identification of *inlG* and *inlH* brings the number of members of the internalin multigene family in *L. monocytogenes* to a total of nine, but the results obtained in this study indicate the occurrence of strain-specificity concerning internalins. Interestingly, over 40 different genes encoding proteins with signal peptide and LPXTG motif were detected in the *L. monocytogenes* strain EGDe (The European *Listeria* Genome Consortium). Most of these proteins contain LRRs, thereby highly increasing the number of members of the internalin multigene family. The function of these proteins is yet unknown. Therefore, it will be most important to further study the possible variability in the structure and expression of internalins and internalin-related proteins and their impact on invasion of different cell types and on *in vivo* virulence in appropriate animal models.

The generation of *inlH* by a deletion event which leads to an in-frame fusion of the 5'-portion of *inlC2* with the 3'-terminal part of *inlD* is the first evidence for the variability of internalin genes in *L. monocytogenes* by intergenic recombination. The generation of variable surface proteins due to homologous recombination of multiple gene copies has been previously demonstrated for the anti-phagocytic M-proteins of pyogenic streptococci (Fischetti, 1989; Podbielski *et al.*, 1994; Haanes and Cleary, 1989) and for the Opa proteins of the pathogenic species *Neisseria gonorrhoeae* and *N. meningitidis* (Malorny *et al.*, 1998; Kupsch *et al.*, 1993). The Opa proteins are involved in adherence to different cells such as to human leucocytes and epithelial cells, depending on the type of Opa proteins expressed (Kupsch *et al.*, 1993). Furthermore, it was shown that expression of these genes can be turned on and off independently. A similar cell tropism was also postulated for the listerial internalin multigene family (Dramsi *et al.*, 1995). However, this is apparently not the case, since some internalins seem to interact with each other and the presence of the interacting partners is required for efficient internalization into host cells (Bergmann, Raffelsbauer *et al.*, manuscript submmited).

In addition to *L. monocytogenes*, another internalin multigene family is also present in *L. ivanovii*, the other pathogenic species of the genus *Listeria*. Most of these internalins belong to the class of small internalins. Characteristic for this group is the lack of the the C-terminal part of the molecules comprising the B region and the cell wall anchor. Due to the absence of sequences that can fix the proteins to the bacterial cell surface, these internalins are much smaller ($\leq$ 30 KDa) and found exclusively in the culture supernatant. Whereas InlC is the only small internalin characterized so far in *L. monocytogenes* (Engelbrecht *et al.*, 1996; Domann *et al.*, 1997), four members of this class of internalins, namely i-InlC, i-InlD, i-InlE and i-InlF, were previously described in *L. ivanovii* (Engelbrecht *et al.*, 1998a; Engelbrecht *et al.*, 1998b). In addition, six new small internalin genes, termed i-*inlG*, i-*inlH*, i-*inlI*, i-*inlJ*, i-*inlK* and i-*inlL*, and two *inlB*-related genes, designated as i-*inlB* and i-*inlB2*, were recently detected in this *Listeria* species (Dominguez-Bernal, 2001). The function of the small internalins remains unclear, but *in vivo* studies using the mouse model show that they represent important virulence

factors (Engelbrecht *et al.*, 1996; Engelbrecht *et al.*, 1998b).

Interestingly, *L. monocytogenes* and *L. ivanovii*, the two pathogenic *Listeria* species, possess both the PrfA-regulated virulence gene cluster which enables them a proficient intracellular life cycle (Tilney and Portnoy, 1989; Karunasagar *et al.*, 1993; Lampidis *et al.*, 1994; Gouin *et al.*, 1994). However, these bacteria differ considerably in their internalin equipment. All *inl* genes of *L. monocytogenes* characterized so far belong to the group of large, surface-bound internalins, with exception of *inlC*. In contrast, more small, secreted internalins are present in *L. ivanovii* and there is no evidence for the presence of large internalins in this species, except the two *inlB*-related genes detected recently. This variation in the internalin equipment may be responsible for the differences observed in host range and pathogenesis of these *Listeria* species.

Most LRR proteins are found in eukaryotes, but there are also a few other examples in bacteria. In addition to the genus *Listeria*, three other pathogenic bacteria contain LRR proteins: YopM of *Yersinia pestis* (Leung and Straley, 1989), IpaH of *Shigella flexneri* (Hartman *et al.*, 1990) and SspH of *Salmonella typhimurium* (Miao *et al.*, 1999). YopM inhibits platelet aggregation by binding thrombin, which normally binds to the GPIb receptor, another LRR protein (Leung and Straley, 1989; Leung *et al.*, 1990). The function of IpaH is unknown, but this protein is an immunodominant antigen during shigellosis. SspH1 and SspH2 were recently identified as important virulence factors of *S. typhimurium* in the calf infection model, although their function could not be exactly determined (Miao *et al.*, 1999). Interestingly, the *ipaH* gene is found in multiple copies on the chromosome and virulence plasmid of *S. flexneri* (Buysse *et al.*, 1987; Hartman *et al.*, 1990), and might therefore constitute another family of LRR proteins in this facultative intracellular pathogen whose intracellular replication cycle resembles that of *L. monocytogenes*.

In the present study, two different methods were used to investigate transcription of the genes *inlG*, *inlH* and *inlE*. First, a GFP based reporter system was applied to measure promoter activity. The regulatory regions upstream of these genes were fused to *gfp* in the reporter plasmid pLSV16-*gfp* and the GFP-mediated fluorescence emitted by listeriae grown extra- or intracellularly was determined using a fluorimeter or a fluorescence activated cell sorter (Raffelsbauer *et al.*, 1998). To compare the activity of the promoters of the *inlGHE* gene cluster to that of other genes, the virulence genes *inlA*, *actA*, and *hly* were used. In a subsequent study, the semi-quantitative RT-PCR technique was used to measure the steady-state amounts of transcripts of different *inl* genes synthesized extracellularly in various time-points of the growth curve. The isolated total RNA was subjected to RT-PCRs using appropriate dilutions of cDNA under insaturated conditions in order to reveal even slight differences in the amount of transcripts. Furthermore, to ensure that equivalent amounts of total RNA were applied, the constitutively expressed *sod* gene was used as internal standard. While the RT-PCR technique measures the steady-state level of transcripts, the GFP-mediated fluorescence determines the promoter activity averaged over a period of time. Both procedures provide a measure of up-regulation of transcription. The RT-PCR technique also allows detection of down-regulation of transcription. However, it is difficult to measure a down-regulation using the GFP method due to the stability of GFP, which in addition is distributed to the daughter cells during cell division.

Both methods applied to study transcription of the genes *inlG*, *inlH* and *inlE* yielded

comparable results. All three genes are transcribed, albeit at low levels compared to other virulence genes (Raffelsbauer *et al.*, 1998 and this study). Therefore, they are not pseudogenes. As revealed by both GFP expression and RT-PCR, the *inlG* promoter is under extracellular growth conditions the most active promoter of the *inlGHE* cluster and transcription of the two remaining genes *inlH* and *inlE* is significantly lower. In front of all three *inl* genes, and especially upstream of *inlG*, there are several putative promoter sequences which could serve as start sites for transcription of each of these genes. In addition, the DNA sequences of the intergenic regions between *inlG* and *inlH*, and between *inlH* and *inlE* also contain putative rho-independent transcriptional terminators, suggesting the monocistronic nature of the three *inl* gene transcripts. RT-PCRs to detect a polycistronic expression of the cluster failed to give significant products, except a faint but reproducible band derived from an *inlG-inlH* bicistronic transcript. However, one cannot exclude a potential polycistronic transcription of *inlH* and *inlE* from the *inlG* promoter at levels below those detectable by the applied method. An example of polycistronic transcription of *inl* genes provides the *inlAB* gene cluster. The genes *inlA* and *inlB* form an operon, whose transcription starts from four promoters located upstream from *inlA* and results in monocistronic *inlA* or bicistronic *inlAB* transcripts (Dramsi *et al.*, 1995; Lingnau *et al.*, 1995; Bohne *et al.*, 1996). However, an *inlB* monocistronic transcript starting in front of *inlB* was also reported (Lingnau *et al.*, 1995).

Transcription of the *inlAB* operon is only in part under the control of PrfA, since only one of the four promoters in front of *inlA* is regulated by this transcriptional activator which regulates expression of most virulence genes (Lingnau *et al.*, 1995; Bohne *et al.*, 1996). The PrfA-regulated promoter of the *inlAB* operon shows in its PrfA-binding site (PrfA box) two mismatches compared to the consensus sequence TTAACANNTGTTAA. It was previously suggested that mismatches within PrfA binding sites reduce the affinity of this transcriptional regulator to the promoter (Freitag and Portnoy, 1994). There is no evidence for a conserved PrfA-box in front of any of the genes *inlG*, *inlH* and *inlE*, and expression of GFP under the control of the expression sites of these genes occurs with the same intensity in a *prfA* deletion mutant and in a strain complemented with additional copies of *prfA* as in the wild type. These data suggest that transcription of the genes *inlG*, *inlH* and *inlE* is not dependent on PrfA. Nevertheless, a putative PrfA binding site could be detected in the promoter region of *inlE* which shows three mismatches in comparison to the perfect PrfA box.

In contrast to the new large internalin genes, transcription of *inlC* is fully dependent on PrfA (Engelbrecht *et al.*, 1996). The *inlC* gene has only one promoter which is strictly PrfA-dependent, since expression of InlC is not detectable in the *prfA* deletion mutant and is increased in a strain complemented with additional copies of PrfA (Engelbrecht *et al.*, 1996). A similar PrfA-dependent transcriptional regulation is also observed in the other small internalin genes described from *L. ivanovii* (Engelbrecht *et al.*, 1998a; Engelbrecht *et al.*, 1998b) and in the virulence genes *hly*, *plcA*, *actA* and *plcB* (Leimeister-Wächter *et al.*, 1990; Bohne *et al.*, 1994; Bohne *et al.*, 1996). The products of these genes are required for the intracellular replication cycle (reviewed by Cossart and Lecuit, 1998; Goebel and Kuhn, 2000). The release of the bacteria from the host cell phagosome is mediated by the products of *hly* (LLO) and *plcA* (PlcA). LLO (listeriolysin O), a pore-forming hemolysin, is activated in acid environments such as the phagosome. PlcA is

a phosphatidylinositol-specific phospholipase C also involved in lysis of the phagosomal membrane (Camilli *et al*., 1993). Intracellular movement in the host cell cytoplasm and cell-to-cell spread is mediated by ActA, a listerial surface protein which recruits and polymerizes cellular actin, allowing listeriae to move (Domann *et al*., 1992; Kocks *et al*., 1992). In addition to ActA, spreading from cell to cell also requires LLO and PlcB, a second phospholipase C with lecithinase activity (Gedde *et al*., 2000; Vazquez-Boland *et al*., 1992). The later proteins are responsible for the lysis of the double membrane which involves the spreading bacteria. Recent studies showed that transcription of these genes is induced within the host cells (Bubert *et al*., 1999; Moor *et al*., 1999), which is in agreement with their function in the intracellular replication cycle. Furthermore, it could be shown that this induction is differential and dependent on the time post-infection and the location of the bacteria within the host cells. Consequently, the promoters of *hly* and *plcA* are predominantly induced within the phagosomal compartment, whereas that of *actA* is activated in the host cell cytosol (Bubert *et al*., 1999). A similar induction of gene transcription under intracellular conditions or when bacteria are incubated in the presence of mammalian cells could not be detected with the genes *inlG*, *inlH* and *inlE* using the sensitive GFP based expression system (Raffelsbauer *et al*., 1998 and this study), suggesting that transcription of these genes occurs predominantly extracellularly, in contrast to other known PrfA-regulated virulence genes (Bubert *et al*., 1999). Similarly to the genes *inlG*, *inlH* and *inlE*, the *inlAB* operon is only poorly transcribed within macrophage-like and epithelial cells (Bubert *et al*., 1999). In both cell types transcription was lower than under extracellular growth conditions in rich culture media. This is in agreement with the assumption that the *inlA* and *inlB* gene products are not required during intracellular bacterial growth. In contrast, transcription of *inlC* is induced intracellularly (Engelbrecht *et al*., 1996). Using a semi-quantitative RT-PCR assay, an induction of transcription of *inlC* was detected within J774 macrophages and reached a maximum approx. 5 to 6 h post-infection, at time-point when bacteria begin to spread from cell to cell. However, the *inlC* mutant was not affected in the ability to spread, neither in homolog monolayers of epithelial Caco-2 cells or J774 macrophages (Engelbrecht *et al*., 1996), nor in heterologous plaque assays using macrophages and several non-phagocytic cells as targets (Greiffenberg *et al*., 1998).

Expression levels of PrfA-regulated genes are rather low when bacteria are grown in rich culture media. Only the genes *hly* and *plcA*, which are required at early stages of the bacterial intracellular cycle, are expressed extracellularly in considerable amounts. However, transcription of *prfA* and most PrfA-regulated genes can be induced upon a shift from rich into minimal essential medium MEM (Bohne *et al*., 1994; Bohne *et al*., 1996). In this medium, transcription of the *actA–plcB* operon is induced most efficiently, whereas that of *hly* is only slightly induced. Interestingly, transcription of *inlA* is rather repressed after this shift (Bohne *et al*., 1996), while that of *inlC* and the other small internalin genes of *L. ivanovii* is induced under these conditions (Engelbrecht *et al*., 1996; Engelbrecht *et al*., 1998b). Recent studies showed that in MEM PrfA is converted into a form that has a higher affinity to the PrfA-binding sites of PrfA-regulated genes (Böckmann *et al*., 1996). The *inlG*, *inlH* and *inlE* genes are not activated after a shift into MEM, which is in line with their PrfA-independence (Raffelsbauer *et al*., 1998). A downregulation of gene expression similar to that observed with InlA in Northern blots could not be observed with

the GFP expression system, probably due to the high stability of GFP.

The role of the internalins InlA and InlB in mediating invasion of *L. monocytogenes* into mammalian cells *in vitro* has been unequivocally demonstrated (reviewed by Kuhn and Goebel, 2000). However, the importance of these invasins in bacterial virulence *in vivo* has been under debate for many years. It was previously shown that oral infection of BALB/c mice with an in-frame *inlAB* deletion mutant results in similar numbers of viable bacteria of the mutant compared to the wild type in liver, spleen and lymph nodes (Dramsi *et al*., 1995). Since InlA is necessary for invasion of epithelial Caco-2 cells (Gaillard *et al*., 1991), these results questioned the role of this internalin *in vivo* and suggested either the existence of gene redundancy or alternative ways to pass the intestinal epithelium and infect underlying cells and tissues. This was confirmed later as the *inlAB* mutant was shown to translocate from the gut lumen to deeper organs as efficiently as the parental strain after inoculation of ligated intestinal loops (Pron *et al*., 1998). In the previous study (Dramsi *et al*., 1995), a reduction of colony forming units (CFU) of the *inlAB* mutant compared to the wild type in liver on day 3 post-infection was detected, which argues for a role of the *inlAB* gene locus in hepatocyte invasion *in vivo*. Similar results were obtained with an *inlAB* transposon mutant, which was completely eliminated from liver on day 4 p. i. (Gaillard *et al*., 1996). Since these studies used *inlAB* double mutants, they could not determine the *in vivo* role of InlA and InlB separately. Another study using single mutants reported that both *inlA* and *inlB* mutants are strongly attenuated for virulence in the mouse model, since they yielded reduced numbers of viable bacteria in liver and spleen 1, 3 and 6 days after intraperitoneal injection (Lingnau *et al*., 1995). These findings were however not confirmed by subsequent investigations which showed that the absence of the *inlAB* gene products did not affect the ability of *L. monocytogenes* to invade hepatic cells and colonize the liver of infected animals (Gregory *et al*., 1996). The controversial data concerning the role of InlA *in vivo* were recently elucidated by studies which show that murine E-cadherin, in contrast to human and guinea pig E-cadherins, does not interact with InlA (Lecuit *et al*., 1999; Lecuit *et al*., 2001). Furthermore, in guinea pigs and transgenic mice expressing human E-cadherin, InlA was found to mediate invasion of enterocytes and crossing the intestinal barrier, revealing the importance of this protein *in vivo* and excluding the mouse as model organism for studying the function of InlA (Lecuit *et al*., 2001). Whether the mouse model is appropriate to study the role of other internalins, whose receptors are unknown, requires further investigations.

No decrease of virulence in BALB/c mice after i. v. inoculation was reported for mutants lacking the *inlC2DE* gene cluster (Dramsi *et al*., 1997). In addition, the LD_{50} of an *inl* mutant lacking *inlA* to *inlF* was similar to that of *inlAB*, which was only slightly increased in comparison to the wild type strain. These results suggest that the genes *inlC2*, *inlD*, *inlE* and *inlF* do not play a role in bacterial virulence. When the number of viable bacteria in liver and spleen was determined 3 days after i. v. injection of strains lacking *inlC2DE* either alone or in combination or *inlA-inlE* or *inlA-inlF*, no differences could be detected between these mutants and the parental strain (Dramsi *et al*., 1997). This is intriguing since none of the two later mutants showed a decrease in bacterial counts in the liver similar to that which had been observed previously with the *inlAB* mutant (Dramsi *et al*., 1995).

In contrast to the *inlC2DE* mutant, the Δ*inlGHE* mutant showed a significant re-

duction of up to 3 log units of viable bacteria in liver and spleen after oral infection of C57BL/6 mice in comparison to the isogenic wild type strain (Raffelsbauer *et al.*, 1998). This notable reduction of bacterial counts in liver and spleen suggests that the *inlGHE* deletion mutant is impaired in the proficient entry and/or replication in these two organs and argues for an important role of the *inlGHE* gene cluster in virulence. Similar reductions in virulence were also observed with strains defective in the small internalins *inlC* of *L. monocytogenes* and i-*inlE* and i-*inlF* of *L. ivanovii* (Engelbrecht *et al.*, 1996; Engelbrecht *et al.*, 1998b). When injected intravenously in mice, the LD_{50} of the *inlC* deletion mutant was increased by 1.5 log units, indicating that InlC contributes to virulence in the mouse model (Engelbrecht *et al.*, 1996). This contribution was even more pronounced in mutants lacking i-*inlE*, i-*inlF* or i-*inlFE*, which failed to kill C57BL/6 mice even at high i. v. infectious doses of 10^9 bacteria per mouse.

It was previously reported that InlA is necessary for entry into Caco-2 cells (Gaillard *et al.*, 1991; Dramsi *et al.*, 1995), while InlB is required for entry into other cell lines, including the epithelial cell lines HeLa, CHO and Vero (Ireton *et al.*, 1996), the hepatocytic cell lines HepG-2 and TIB73 (Dramsi *et al.*, 1995), and especially the endothelial cell lines HBMEC and HUVEC (Greiffenberg *et al.*, 1998; Parida *et al.*, 1998). However, another report showed that both InlA and InlB are required for invasion into the epithelial cell lines Caco-2, HeLa, PtK2 and HEp-2 (Lingnau *et al.*, 1995). In addition, in order to mediate entry into HepG-2 cells, InlB needs the supportive function of InlA (Dramsi *et al.*, 1995). Deletion of the *inlAB* operon abolishes the ability of *L. monocytogenes* to invade non-phagocytic cells, suggesting that InlA and InlB are the only invasins of this facultative intracellular bacterium. Indeed, previous studies showed that latex beads coated with purified InlA or InlB proteins and non-invasive bacteria expressing these proteins can invade several cell types, indicating that these internalins are sufficient to promote internalization of *L. monocytogenes* into non-phagocytic cells (Gaillard *et al.*, 1991; Mengaud *et al.*, 1996; Lecuit *et al.*, 1997; Braun *et al.*, 1998). However, the rate of internalization in these studies, when determined, was very low and difficult to compare to the situation using *L. monocytogenes* wild type. When tested in cellular invasion assays using the cell lines Caco-2, TIB73, HepG-2 and S180 the invasion rates of *inlC2DE* single and triple mutants were not reduced compared to the wild type, indicating that these internalins are not required for entry in these cell lines (Dramsi *et al.*, 1997).

To study whether the internalins InlG, InlH and InlE are also involved in invasion of *L. monocytogenes* EGD into mammalian cells, mutants carrying chromosomal in-frame deletions in the *inlGHE* gene cluster and in other *inl* genes were constructed in the present study. Construction of the *inl* mutants was performed using the mutagenesis vector pLSV1 (Wuenscher *et al.*, 1991), which has been successfully used in our laboratory to construct several mutants (Engelbrecht *et al.*, 1996; Engelbrecht *et al.*, 1998b). The shuttle vector pLSV1 carries a gene for erythromycin resistance and a temperature-sensitive origin for Gram-positive bacteria. The strategy used for deletion is based on homologous recombination and applies the use of erythromycin as selective marker and incubation of bacteria at a non-permissive temperature of 42°C. The use of these mutants in cellular invasion assays performed in a doctoral thesis by B. Bergmann could reveal that, in contrast to InlA and InlB, the internalins InlG, InlH and InlE are not invasins by themselves. Deletion of the complete *inlGHE* gene cluster or of the single genes

inlG, *inlH* and *inlE* leads to an increase in invasiveness of *L. monocytogenes* into all non-phagocytic cells tested, indicating that the products of these genes do not trigger but rather inhibit internalization of the bacteria into non-phagocytic mammalian cells (this study and Bergmann, Raffelsbauer *et al.*, manuscript submmited). The increase in invasiveness was abolished when the single mutants were reverted to the wild type genotype by inserting a copy of the respective wild type genes into the chromosome, demonstrating that the enhanced invasiveness was specifically caused by the deletion of the corresponding *inl* gene (Bergmann, Raffelsbauer *et al.*, manuscript submmited).

The increase in invasiveness observed with the Δ*inlGHE* mutant seems to be specific for non-phagocytic cells, since this mutant was internalized into professional phagocytes of the cell line P388 as efficiently as the wild type in cellular invasion assays *in vitro* in the absence of serum. Under these conditions uptake of *L. monocytogenes* by phagocytes was shown to depend, at least to a low extent, on the invasion factors InlA, p60 and ActA (Hess *et al.*, 1995; Sawyer *et al.*, 1996; Alvarez-Dominguez *et al.*, 1997), and additionally on lipoteichoic acid, which was shown to bind to the macrophage scavenger receptor (Greenberg *et al.*, 1996). These data suggest that in the absence of opsonins *L. monocytogenes* uses a pathway to enter professional phagocytes which involves in part the same invasins required for entry into non-phagocytic cells. Interestingly, in the presence of serum, uptake of opsonized *L. monocytogenes* by macrophages is mediated by the complement receptor 3 (CR3) of the complement component C3 (Drevetts and Campbell, 1991) and is mostly independent of internalins. This C3-CR3 interaction leads to an efficient killing of intracellular listeriae by the macrophages. Besides C3 the complement component C1q was also shown to be involved in phagocytosis of *L. monocytogenes* by macrophages and this C1q-C1qR pathway seems to be relevant *in vivo* (Alvarez-Dominguez *et al.*, 1993).

It was previously shown that the *inlB* mutant is able to adhere to the endothelial cells HBMEC as efficiently as the wild type strain, suggesting that InlB is not necessary for adherence of *L. monocytogenes* to these cells (Greiffenberg *et al.*, 2000). Also the apathogen non-invasive species *L. innocua* is able to adhere to HBMEC (Greiffenberg *et al.*, 2000). Since this bacterium seems to lack internalins, it was postulated that adhesion of *L. monocytogenes* at least to HBMEC does not require internalins. Indeed, several bacterial surface proteins other than internalins have been previously identified which are necessary for adhesion of *L. monocytogenes* to mammalian cells, such as p60, ActA, Ami, p104, also called LAP, and different fibronectin binding proteins (Kuhn and Goebel, 1989; Alvarez-Dominguez *et al.*, 1997; Milohanic *et al.*, 2000; Pandiripally *et al.*, 1999; Gilot *et al.*, 1999). It is interesting that two of these adhesins, namely p60 and Ami, are autolysins (Wuenscher *et al.*, 1993; Braun *et al.*, 1997; Milohanic *et al.*, 2001). Expression of LAP (*Listeria* adhesion protein) is low during the exponential but high during the stationary growth phase of bacteria grown at 37 or 42°C, but not at 25°C, suggesting a modulation of the LAP expression by the growth temperature (Santiago *et al.*, 1999). Although this expression varies depending on the temperature and growth phase, enhanced expression does not result in increased invasiveness, probably because a few LAP molecules are sufficient to mediate adherence to Caco-2 cells. Surprisingly, the *inlGHE* mutant shows a three- to four-fold enhanced ability to adhere to confluent Caco-2 monolayers, in contrast to the *inlA* mutant, which adheres only poorly to these differentiated Caco-2 cells (Karunasagar and Raffelsbauer, unpublished data). A dependence of adherence to

Caco-2 cells on InlA has been observed before (Gaillard *et al.*, 1991), suggesting that InlA not only mediates internalization but also adhesion to these cells. The efficiency of internalization of *L. monocytogenes* into Caco-2 cells was shown to depend on the state of polarization and differentiation of the cells, decreasing substantially when Caco-2 monolayers grown beyond confluency are used (Gaillard and Finlay, 1996). It was previously reported that invasion of *L. monocytogenes* into polarized, differentiated Caco-2 cells from the apical side involves the interaction of cellular microvilli with the bacterial surface (Karunasagar *et al.*, 1994). This process seems to be less efficient than invasion of non-differentiated Caco-2 cells, which occurs via the zipper mechanism and predominantly at the basolateral side (Mengaud *et al.*, 1996). This is in line with the observation that E-cadherin, the receptor for InlA, is located at this side of the cell (Mengaud *et al.*, 1996; Hermiston and Gordon, 1995). The InlA-dependent adherence of *L. monocytogenes* to differentiated Caco-2 cells seems to be supported by InlC, since the Δ*inlA/C* mutant was nearly impaired to adhere to these cells (Karunasagar and Raffelsbauer, unpublished data).

The existence of an *inlAB*-independent invasion pathway has been postulated. A previous report showed that fibroblasts of the cell lines WI-38 and HEL 299 are permissive for *inlA* and *inlB* deletion mutants, suggesting that there exist other factors which can mediate invasion of *L. monocytogenes* into non-phagocytic cells (Lingnau *et al.*, 1995). It was also demonstrated that a protein other than internalins is involved in adhesion and invasion of *L. monocytogenes* into fibroblasts of the cell line 3T6 (Kuhn and Goebel, 1989). This protein, termed p60, is encoded by the *iap* (invasion associated protein) gene and has in addition a second function as a murein hydrolase involved in septum separation (Wuenscher *et al.*, 1993). Expression of the *iap* gene is independent of PrfA and regulated at the posttranslational level by a still unknown mechanism (Köhler *et al.*, 1991). Spontaneous p60 mutants which produce reduced levels of this protein form long cell chains which are non-invasive (Kuhn and Goebel, 1989). It is however unclear whether p60 mediates adherence and invasion by binding to a specific cellular receptor or whether this strongly basic protein simply neutralizes negative charges on the host cell surface, thereby allowing a better bacterial adhesion. P60 is a secreted protein mostly found in the culture supernatant, but a small portion of the protein is also present on the bacterial cell surface (Ruhland *et al.*, 1993). It was recently shown that secreted p60 specifically binds to the cell membrane of Caco-2 cells in a uniform distribution (Park *et al.*, 2000). This suggests the existence of specific host cell receptors for p60. This interaction may elicit a cellular signal that triggers internalization of *L. monocytogenes* into Caco-2 cells. Whether cell-bound p60 is also able to bind to mammalian cells is yet unknown. Interestingly, p60 mutants were shown to adhere normally to Caco-2 cells and to be invasive after disruption of the bacterial cell chains by ultrasonication (Bubert *et al.*, 1992), suggesting that p60 is not necessary for efficient bacterial adherence and that the mechanism of action of this protein might be more complex and involve interactions with host cells. A previous work suggested that also ActA may play a role in adhesion and invasion into mammalian cells (Alvarez-Dominguez *et al.*, 1997). ActA is responsible for the recruitment and polymerization of actin filaments that enable listeriae to move in the cytosol and spread from cell to cell (Domann *et al.*, 1992; Kocks *et al.*, 1992). This large surface-bound protein seems in addition to mediate adhesion of *L. monocytogenes* to mammalian

cells by binding to heparan sulfate proteoglycan (Alvarez-Dominguez *et al.*, 1997). Recently, it was shown that an *actA* deletion mutant is impaired to invade endothelial cells of the HBMEC line (Greiffenberg, 2000). The involvement of a protein encoded by a PrfA-regulated gene from the virulence gene cluster in listerial invasion was previously postulated based on the observation that a *L. monocytogenes* mutant lacking both the *prfA* operon and the *inlA* gene shows reduced invasiveness in mammalian cells (Kuhn *et al.*, 1997; Engelbrecht *et al.*, 1996).

The hypothesis that the internalins InlG, InlH and/or InlE are further invasins of the *inlAB*-independent invasion pathway of *L. monocytogenes* could not be confirmed, since deletion of the complete *inlGHE* gene cluster or of the single genes rather stimulates internalization of *L. monocytogenes* by various non-phagocytic cell types more than two fold (this study and Bergmann, Raffelsbauer *et al.*, manuscript submmited). Using the *inl* combination mutants constructed in the present study it could be shown that the InlB-mediated entry of *L. monocytogenes* into mammalian cells does not require other internalins (Bergmann, Raffelsbauer *et al.*, manuscript submmited). In contrast, InlA alone is unable to trigger efficient internalization into Caco-2 cells and needs the support of InlB, InlC, InlG, InlH and InlE. This observation shows that internalization of *L. monocytogenes* into non-phagocytic mammalian cells is more complex than previously assumed and requires the participation of small and large internalins.

The expression of *inlA* and *inlB* is only in part dependent on PrfA and there is evidence that the two PrfA-independent promoters in front of *inlA* play major roles in expression of both genes under extracellular conditions (Lingnau *et al.*, 1995; Dramsi *et al.*, 1993). In addition, expression of the *inlAB* operon is also modulated by the growth state and by the temperature, being maximal during the logarithmic phase and at 37°C. The PrfA-independent transcription of the three new genes *inlG*, *inlH* and *inlE* argues for their predominant expression under extracellular conditions (Raffelsbauer *et al.*, 1998). The absence of expression of these genes when the bacteria grow within host cells is in line with this assumption. Furthermore, the genes *inlG*, *inlH* and *inlE* are not activated after bacterial contact to cells of the HepG-2 cell line, whose internalization is dependent on both InlA and InlB proteins. Whether these genes are expressed *in vivo* is unknown. Up to now, *in vivo* expression of listerial virulence factors has been demonstrated only for listeriolysin, InlC, InlB and ActA (Grenningloh *et al.*, 1997). Transcription of the genes *inlC2*, *inlD* and *inlE* of BHI grown bacteria was shown to occur at an early stage of the exponential growth phase and seems to be transiently and less efficient than that of the *inlAB* operon (Dramsi *et al.*, 1997). Similarly, the intensity of transcription of the genes from the *inlGHE* gene cluster is lower than that of the *inlA* and *inlB* genes (Raffelsbauer *et al.*, 1998 and this study).

The present study shows that deletion of the *inlGHE* gene cluster induces transcription of the *inlA* and *inlB* genes, but not of *inlC* (Bergmann, Raffelsbauer *et al.*, manuscript submmited). In addition, deletion of *inlA* enhances transcription of *inlB* and vice-versa. A reduction of expression of *inlA* in the presence of the *inlB* gene was previously demonstrated in a *L. innocua* strain carrying plasmids containing either *inlAB* or *inlA* (Dramsi *et al.*, 1993). The enhanced level of *inlA* and *inlB* transcripts is not solely responsible for the increase in invasiveness of the *inlGHE* mutant, since the single mutants *inlG*, *inlH* and *inlE* also show increased invasion rates although transcription of the *inlA* and *inlB* genes

in these mutants is not altered (Bergmann, Raffelsbauer *et al*., manuscript submmited). This suggests the existence of a mechanism that regulates transcription of the large *inl* genes. An up-regulation requires deletion of all three internalins, whereas deletion of one single gene suffices to enhance the invasion ability.

Recently, it was shown that expression of the genes *inlA*, *inlB* and *actA* is modulated by ClpC, an ATPase involved in virulence of *L. monocytogenes* by promoting early bacterial escape from the phagosomal compartment of macrophages (Nair *et al*., 2000). The reduced expression of these invasion factors in the *clpC* mutant impaired bacteria to adhere to and invade murine hepatocytes *in vivo* and *in vitro*. This ClpC-dependent modulation occurs at the transcriptional level, since RNA slot blot analysis using the *clpC* mutant revealed a reduction of transcription of *inlA*, *inlB* and *actA*, but not *prfA*. Therefore, expression of virulence genes in *L. monocytogenes* seems to be regulated by a more complex mechanism and involved new alternative transcriptional regulators in addition to PrfA. The participation of Clp chaperones in the modulation of virulence factors has been previously proposed. This is the case for ClpP, a subunit of the Clp protease which modulates expression of Ail, a cell surface protein involved in adhesion and invasion of *Yersinia enterocolitica* (Pederson *et al*., 1997). Recently another chaperone, SicA, belonging to the type III secretion system of *Salmonella typhimurium* was shown to act together with InvF in the activation of expression of the *Salmonella* invasion protein SigD (Darwin and Miller, 2000).

No effect on virulence was observed when mice were infected i. v. with mutants harboring single or combinational deletions in *inlC2*, *inlD* and *inlE* (Dramsi *et al*., 1997). In contrast, the present study revealed that deletion of the *inlGHE* gene cluster significantly reduces virulence of *L. monocytogenes* in the mouse model after oral infection (Raffelsbauer *et al*., 1998). The positioning of the strongest promoter upstream from *inlG* may explain the difference regarding the contribution of this gene cluster to virulence. Since both studies differ in the route of infection, it is also thinkable that the internalins InlG, InlH and InlE are necessary for efficient passage of the gut epithelium. However, the high ability of the *inlGHE* mutant to invade Caco-2 epithelial cells contradicts this hypothesis. Using the GFP expression vector it could be shown that the *inlGHE* mutant is able to replicate and spread in epithelial Caco-2 monolayers as efficiently as the wild type strain (Raffelsbauer *et al*., 1998), indicating that deletion of these internalins does not affect replication and spreading of listeriae between host cells of the same type. However, the ability of the *inlGHE* mutant to spread between different cell types was not investigated. One may speculate that this gene cluster may be involved in heterologous spreading between different cell types during infection *in vivo*, such as between macrophages and hepatocytes, endothelial cells or others. A critical point during systemic listeriosis is the ability of *L. monocytogenes* to survive and replicate in phagocytic cells, in which the bacteria are transported via lymph and blood to distinct organs (Racz *et al*., 1972). Indeed, it was recently demonstrated that infected phagocytes have a major role in dissemination of *L. monocytogenes* during infection in mice by representing a more efficient pathway of infection of organs such as liver and brain than the direct invasion of blood-borne bacteria into hepatocytes or endothelial cells (Drevets, 1999; Drevets *et al*., 2001). The first report shows that inocula of *L. monocytogenes* infected macrophages lead to higher number of viable bacteria in liver than infection with broth-grown bacteria. The present study shows

that the *inlGHE* mutant is taken up by phagocytic cells *in vitro* as efficiently as the wild type. However, a long-term survival and replication of this mutant in these cells have not been yet demonstrated. It is tempting to speculate that the mutant is impaired to replicate within these phagocytes. This could explain the reduced virulence observed with the mutant in the mouse model. However, a more rapid elimination of the mutant bacteria in liver and spleen by cells of the immune system cannot be ruled out formally. Taken together, the enhanced invasiveness of the *inlGHE* mutant into non-phagocytic cells *in vitro* and the reduced virulence observed in the mouse model suggest a more complex function of the internalins InlG, InlH and InlE, which may also involve additional listerial virulence factors including other internalins and transcriptional regulators.

Most *L. monocytogenes* strains, such as the EGD strain used here, cause septicaemia and infections of the central nervous system like meningitis and meningoencephalitis. However, *L. monocytogenes* isolates have been reported which cause a localized disease in form of gastroenteritis (Heitmann *et al.*, 1997; Dalton *et al.*, 1997). In the present study the question was raised whether *L. monocytogenes* gastroenteritis strains are impaired to spread through the organism due to the lack of some internalins, resulting in a form of listeriosis which is restricted to the region of the entry portal, i. e. the gastrointestinal tract. In order to study a putative involvement of the internalins InlG, InlH and InlE in the early bacterial spreading process *in vivo*, the occurrence of the *inlGHE* gene cluster was investigated in three gastroenteritis strains. In addition, strain EGD and three other *L. monocytogenes* clinical isolates which cause sepsis were used for comparison. The present study revealed that at least two of the three gastroenteritis strains tested possess the *inlGHE* gene cluster, excluding a correlation between this gene cluster and the gastroenteritis form of disease. However, the genes *inlG*, *inlH* or *inlE* could not be clearly detected in two sepsis strains. These results give evidence of variations within the sequences of the genes from the *inlGHE* gene cluster among distinct *L. monocytogenes* isolates and suggest the occurrence of different equipments of internalins in these strains.

Eukaryotic cell death occurs either by apoptosis or necrosis. Some bacterial pathogens were shown to be able to induce host cell death (reviewed by Moss *et al.*, 1999; Weinrauch and Zychlinsky, 1999). Activation or prevention of cell death may be a critical factor in the outcome of an infection. Programmed cell death has been observed as response to infection by a large number of animal and plant pathogens. It has been reported that infection of different cell types with *L. monocytogenes* may elicit cell death by apoptosis or necrosis. Most of these studies were performed with professional phagocytes. *L. monocytogenes* infection induces apoptosis *in vivo* in mouse lymphocytes derived from the spleen and lymph nodes (Merrick *et al.*, 1997) and in murine but not in human dendritic cells *in vitro* (Guzmán *et al.*, 1996; Kolb-Mäurer *et al.*, 2000). Interestingly, listeriae infected murine bone marrow-derived macrophages undergo rather necrotic cell death (Barsig and Kaufmann, 1997). Infection with *L. monocytogenes* also did not cause apoptosis of J774 macrophages (Zychlinsky *et al.*, 1992). In contrast, *Shigella flexneri* has been shown to kill murine macrophages by inducing apoptosis (Zychlinsky *et al.*, 1992). Induction of programmed cell death is dependent on the *Shigella* protein IpaB, which directly binds and activates the interleukin-1β-converting enzyme ICE, also called caspase-1 (Zychlinsky *et al.*, 1994; Chen *et al.*, 1996). A mechanistic cognate of IpaB has not yet been identified in *L. monocytogenes* (Barsig and Kaufmann, 1997). Interestingly, *S. flexneri*

fails to induce cell death in epithelial cells, as this cell type represents a habitat for this enterobacterium (Mantis *et al.*, 1996). Thus, bacterial pathogens not only use different strategies to kill mammalian cells by apoptosis or necrosis, but they are also able to do this in a cell type-specific way.

Whereas induction of cell death in phagocytes by *L. monocytogenes* has been studied to some extent, very little is known about cell viability of non-phagocytic cells after infection with this intracellular bacterium. Up to now, induction of apoptosis in non-phagocytic cells by *L. monocytogenes* has been reported only for hepatocytes, the major replication site of the bacteria *in vivo* (Rogers *et al.*, 1996). It has been postulated that suppression of the cell death pathway may facilitate the proliferation of intracellular pathogens (Weinrauch and Zychlinsky, 1999). Thus, induction of cell death may inhibit pathogen dissemination. Therefore it was supposed that a localized *L. monocytogenes* infection in form of gastroenteritis observed with some isolates may be due to cell death at the portal of entry (the gastrointestinal tract), which impairs the bacteria to proliferate and disseminate to distant organs.

In the present study, the ability of different *L. monocytogenes* clinical isolates and internalin mutant strains to induce cell death in epithelial Caco-2 and macrophage-like J774 cells was investigated by detection of DNA fragmentation, which is one of the hallmarks that characterizes apoptosis (Wyllie, 1980; Duke, 1983). This study clearly shows that *L. monocytogenes* strain EGD does not induce apoptosis in both cell lines under the conditions used. This result is in accordance with previous reports (Zychlinsky *et al.*, 1992) and with microscopical studies using the dyes Hoechst 33342 and propidium iodide which showed that infection of Caco-2 cells with strain EGD leads to an intensive spreading of the bacteria without signs of significant cell death (Wagner *et al.*, manuscript in preparation). In contrast, considerable differences were detected among the distinct *L. monocytogenes* clinical isolates concerning their ability to induce cell death in comparison to strain EGD. Both gastroenteritis and sepsis causing *L. monocytogenes* isolates are able to induce apoptosis, albeit to different extents. In Caco-2 cells, the highest levels of DNA fragments were detected with the isolates 535 (sepsis strain) and 1724 (gastroenteritis strain). The second gastroenteritis strain 1727 is also able to induce apoptosis, but at a lower intensity. Sepsis strains 535, 579 and EGD differ considerably in their ability to induce cell death. Whereas isolate 535 is able to efficiently kill cells through apoptosis, 579 behaves rather like strain EGD and does not differ significantly from uninfected control cells. Therefore, effect on cell viability alone cannot explain a localization of bacteria during the gastrointestinal form of listeriosis, at least not using the epithelial cell line Caco-2 as model. A correlation could be observed between the amount of DNA fragments detected in Caco-2 cells and the bacterial invasiveness of the strains tested (Wagner *et al.*, manuscript in preparation), indicating that induction of apoptosis in these cells is proportional to the number of internalized bacteria, as reported in a recent study (Valenti *et al.*, 1999). Interestingly, the two-fold increase in DNA fragmentation measured in Caco-2 cells infected with the Δ*inlGHE* mutant also correlates with the two-fold enhanced invasiveness observed with this mutant in this cell line. Whether the differences in DNA fragmentation observed between the strains tested and the control strain EGD are only the reflect of different invasion abilities or are caused by interactions with specific factors requires further investigations.

Material

This Chapter describes the material specifically used in the present study, such as bacterial strains, plasmids, cell lines, commercial kits and oligonucleotides. For chemicals and solutions of more general use in standard genetic, biochemical and cell biological procedures not listed here see the given references or standard protocols.

4.1   Bacterial strains and plasmids

Bacterial strains used in the present study are listed in Tables 4.1, 4.2, 4.3 and 4.4. Most *Listeria monocytogenes* strains used were obtained from the American Type Culture Collection (ATCC; Rockville, Md., USA), from the National Collection of Type Cultures (NCTC; London, England) or from the Special *Listeria* Culture Collection (SLCC; Würzburg, Germany). *L. monocytogenes* EGD was provided by S. H. E. Kaufmann, University of Ulm, Germany. *L. monocytogenes* L99 was obtained from T. Chakraborty, Institute of Medical Microbiology, University of Gießen, Germany. *L. monocytogenes* strain 35264 (H) was provided by H. Hof, Institute of Medical Microbiology and Hygiene, University of Mannheim, Germany. *L. innocua* serotype 6b was taken from our institute's culture collection. Other *L. monocytogenes* wild type strains used are described in the given references (Table 4.1). All *L. monocytogenes* internalin deletion mutants used were constructed in the present study starting from the wild type strain *L. monocytogenes* EGD as described in section 5.2, except the mutants $\Delta inlA$, $\Delta inlB$, $\Delta inlC$, $\Delta inlA/B$ and $\Delta inlB/C$, which were constructed where else according to the given references (Table 4.2). Bacterial strains harboring GFP expression vectors were constructed as described in section 5.3.1 or in Bubert *et al*. (1999) (Table 4.3). *Escherichia coli* DH5α was obtained from Bethesda Res. Labs, Bethesda, MD, USA, and used as cloning host for nucleotide sequence analysis and construction of knock-out and knock-in plasmids (Table 4.4). Bacterial strains were kept in own culture collection at $-80°$C. Designation of each strain in this collection is represented by the number in parentheses beginning with either S, G or C. A list of these strains is included in App. A. Vectors and plasmids used here are listed in Tables 4.5 and 4.6. In general, plasmids were derived from the vectors pUC18 (Pharmacia Biotech), pLSV1 (Wuenscher *et al*., 1991), pLSV16*gfp* (Bubert *et al*., 1999) or pERL3 (Leimeister-Wächter *et al*., 1990). For clearness reasons, plasmids constructed for nucleotide sequence determination are not included in these Tables.

Table 4.1: Bacterial wild type strains used in this study.

Species	Strain	Serotype	Source of reference
Listeria monocytogenes	EGD wild type (S1)	1/2a	S. H. E. Kaufmann
L. monocytogenes	NCTC 7973 (S3)	1/2a	NCTC
L. monocytogenes	SLCC 2755 (S4)	1/2b	SLCC
L. monocytogenes	NCTC 5348 (S5)	1/2c	NCTC
L. monocytogenes	NCTC 5105 (S6)	3a	NCTC
L. monocytogenes	SLCC 5543 (S7)	3b	SLCC
L. monocytogenes	SLCC 2479 (S8)	3c	SLCC
L. monocytogenes	L99 (S9)	4a	T. Chakraborty
L. monocytogenes	SLCC 4013 (S10)	4b	SLCC
L. monocytogenes	ATCC 19116 (S11)	4c	ATCC
L. monocytogenes	ATCC 19117 (S12)	4d	ATCC
L. monocytogenes	ATCC 19118 (S13)	4e	ATCC
L. ivanovii	ATCC 19119	5	ATCC
L. innocua		6b	institute's collection
L. monocytogenes	1724	4b	(Heitmann *et al.*, 1997)
L. monocytogenes	579	4b	(Linnan *et al.*, 1988)
L. monocytogenes	35264 (H)	1/2a	H. Hof
L. monocytogenes	1727	1/2b	(Dalton *et al.*, 1997)
L. monocytogenes	535	4b	(Bille, 1998)
L. monocytogenes	536	4b	(Bille, 1998)

4.2   Media for bacterial growth and antibiotics

Media used for bacterial growth are shown in Table 4.7. *Listeria* spp. strains were grown aerobically in brain-heart-infusion broth (BHI; Difco Laboratories, Detroit Mich. USA; Life Technologies, Karlsruhe, Germany; Scharlau Microbiology, Barcelona, Spain). *E. coli* strains were grown in 2× YT broth. Agar plates were made by adding 1.5% agar to the media, i. e. 15 g agar in 1 liter broth. For the growth of bacterial strains containing plasmids, antibiotics were added to the media as shown in Table 4.8. More detailed information about bacterial growth conditions is given in section 5.4.1.

4.3   Mammalian cell lines and media for cell culture

In this study following mammalian cell lines were used:

Caco-2 cells: ATCC HTB-37, human colon epithelial cells (Adenocarcinoma), subclone 1.

J774 cells: ATCC TIB-67, murine macrophages.

P388 cells: ATCC TIB-63, murine macrophages.

TIB73 cells: ATCC TIB-73, murine hepatocytes.

HepG-2 cells: ATCC HB 8065, human hepatocytes.

Table 4.2: Bacterial mutant strains.

Species	Strain	Serotype	Source
L. monocytogenes	EGD Δ*inlGHE* (S14)	1/2a	This study
L. monocytogenes	EGD Δ*inlGHE* (S57)	1/2a	This study
L. monocytogenes	EGD Δ*inlGHE* (S58)	1/2a	This study
L. monocytogenes	EGD Δ*inlGHE* (S59)	1/2a	This study
L. monocytogenes	EGD Δ*inlG*I (S37)	1/2a	This study
L. monocytogenes	EGD Δ*inlG*I (S38)	1/2a	This study
L. monocytogenes	EGD Δ*inlG*II (S53)	1/2a	This study
L. monocytogenes	EGD Δ*inlG*II (S54)	1/2a	This study
L. monocytogenes	EGD Δ*inlH* (S41)	1/2a	This study
L. monocytogenes	EGD Δ*inlH* (S42)	1/2a	This study
L. monocytogenes	EGD Δ*inlE* (S39)	1/2a	This study
L. monocytogenes	EGD Δ*inlE* (S40)	1/2a	This study
L. monocytogenes	EGD *inlG*I$^+$ (S61)	1/2a	This study
L. monocytogenes	EGD *inlG*II$^+$ (S62)	1/2a	This study
L. monocytogenes	EGD *inlG*II$^+$ (S63)	1/2a	This study
L. monocytogenes	EGD *inlH*$^+$ (S79)	1/2a	This study
L. monocytogenes	EGD *inlH*$^+$ (S80)	1/2a	This study
L. monocytogenes	EGD *inlE*$^+$ (S60)	1/2a	This study
L. monocytogenes	EGD *inlE*$^+$ (S69)	1/2a	This study
L. monocytogenes	EGD *inlE*$^+$ (S70)	1/2a	This study
L. monocytogenes	EGD Δ*inlA* (S18 and S64)	1/2a	(Greiffenberg *et al*., 1997)
L. monocytogenes	EGD Δ*inlB* (S65)	1/2a	(Greiffenberg *et al*., 1997)
L. monocytogenes	EGD Δ*inlC* (S19 and S66)	1/2a	(Engelbrecht *et al*., 1996)
L. monocytogenes	EGD Δ*inlA/B* (S20)	1/2a	(Greiffenberg *et al*., 1997)
L. monocytogenes	EGD Δ*inlB/C* (S22)	1/2a	(Greiffenberg *et al*., 1997)
L. monocytogenes	EGD Δ*inlA/C* (S71)	1/2a	This study
L. monocytogenes	EGD Δ*inlA/C* (S73)	1/2a	This study
L. monocytogenes	EGD Δ*inlA/GHE* (S27)	1/2a	This study
L. monocytogenes	EGD Δ*inlA/GHE* (S28)	1/2a	This study
L. monocytogenes	EGD Δ*inlB/GHE* (S67)	1/2a	This study
L. monocytogenes	EGD Δ*inlB/GHE* (S68)	1/2a	This study
L. monocytogenes	EGD Δ*inlC/GHE* (S29)	1/2a	This study
L. monocytogenes	EGD Δ*inlC/GHE* (S30)	1/2a	This study
L. monocytogenes	EGD Δ*inlA/B/GHE* (S34)	1/2a	This study
L. monocytogenes	EGD Δ*inlA/B/GHE* (S35)	1/2a	This study
L. monocytogenes	EGD Δ*inlB/C/GHE* (S36)	1/2a	This study
L. monocytogenes	EGD Δ*inlA/C/GHE* (S75)	1/2a	This study
L. monocytogenes	EGD Δ*inlA/C/GHE* (S76)	1/2a	This study
L. monocytogenes	EGD Δ*prfA* (S15)	1/2a	(Böckmann *et al*., 1996)
L. monocytogenes	EGD wild type × pERL3501 (S16)	1/2a	(Leimeister-W. *et al*., 1990)
L. monocytogenes	EGD Δ*prfA* × pERL3502 (S17)	1/2a	

Table 4.3: Bacterial strains containing GFP expression plasmids.

Species	Strain	Source
L. monocytogenes	EGD wild type × P*inlG-gfp* (G1)	This study
L. monocytogenes	EGD wild type × P*inlH-gfp* (G4)	This study
L. monocytogenes	EGD wild type × P*inlE-gfp* (G7)	This study
L. monocytogenes	EGD Δ*inlA/B* × P*inlG-gfp* (G22)	This study
L. monocytogenes	EGD Δ*inlA/B* × P*inlH-gfp* (G23)	This study
L. monocytogenes	EGD Δ*inlA/B* × P*inlE-gfp* (G24)	This study
L. monocytogenes	EGD Δ*prfA* × P*inlG-gfp* (G2)	This study
L. monocytogenes	EGD Δ*prfA* × P*inlH-gfp* (G5)	This study
L. monocytogenes	EGD Δ*prfA* × P*inlE-gfp* (G8)	This study
L. monocytogenes	EGD wild type × pERL3502 × P*inlG-gfp* (G3)	This study
L. monocytogenes	EGD wild type × pERL3502 × P*inlH-gfp* (G6)	This study
L. monocytogenes	EGD wild type × pERL3502 × P*inlE-gfp* (G9)	This study
L. monocytogenes	EGD wild type × pLSV16 without *gfp* (G20)	This study
L. monocytogenes	EGD Δ*inlGHE* (S14) × pLSV16 without *gfp* (G21)	This study
L. monocytogenes	EGD Δ*inlGHE* (S14) × P*actA-gfp*	This study
L. monocytogenes	EGD wild type × P*inlA-gfp* (G13)	(Bubert *et al.*, 1999)
L. monocytogenes	EGD wild type × P*inlC-gfp* (G19)	(Bubert *et al.*, 1999)
L. monocytogenes	EGD wild type × P*actA-gfp* (G10)	(Bubert *et al.*, 1999)
L. monocytogenes	EGD wild type × P*hly-gfp* (G16)	(Bubert *et al.*, 1999)
L. monocytogenes	EGD Δ*prfA* × P*inlA-gfp* (G14)	(Bubert *et al.*, 1999)
L. monocytogenes	EGD Δ*prfA* × P*hly-gfp* (G17)	(Bubert *et al.*, 1999)
L. monocytogenes	EGD wild type × pERL3502 × P*inlA-gfp* (G15)	(Bubert *et al.*, 1999)
L. monocytogenes	EGD wild type × pERL3502 × P*actA-gfp* (G12)	(Bubert *et al.*, 1999)

Table 4.4: *E. coli* recombinant strains.

Species	Strain	Source of reference
Escherichia coli	DH5α	Bethesda Res. Labs
E. coli	DH5α × pLSVΔ*inlGHE*	(Raffelsbauer, 1997)
E. coli	DH5α × pUC18Δ*inlG*I (G35)	This study
E. coli	DH5α × pLSVΔ*inlG*I (G36)	This study
E. coli	DH5α × pUC18Δ*inlG*II (C4)	This study
E. coli	DH5α × pLSVΔ*inlG*II (C5)	This study
E. coli	DH5α × pUC18*inlG* (C6)	This study
E. coli	DH5α × pLSV*inlG* (C7)	This study
E. coli	DH5α × pUC18Δ*inlH* (C8)	This study
E. coli	DH5α × pLSVΔ*inlH* (C9)	This study
E. coli	DH5α × pUC18Δ*inlE* (C10)	This study
E. coli	DH5α × pLSVΔ*inlE* (C11)	This study
E. coli	DH5α × pLSV*inlH* (C12-C14)	This study
E. coli	DH5α × pLSV*inlE* (C15)	This study
E. coli	DH5α × P*inlG-gfp* (G32)	This study
E. coli	DH5α × P*inlH-gfp* (G33)	This study
E. coli	DH5α × P*inlE-gfp* (G34)	This study
E. coli	DH5α × pLSV1 (G37)	(Wuenscher *et al.*, 1991)
E. coli	DH5α × pERL3502 (G38)	(Leimeister-W. *et al.*, 1990)

Table 4.5: Plasmids used in this study.

Plasmid	Properties	Source
pLSV1	Shuttle vector, 6.3 kb, ColE1-Origin, temperature-sensitive origin (Ori_{ts}) from pEI94, erythromycin resistance gene (erythromycin^R)	(Wuenscher *et al.*, 1991)
pLSVΔ*inlGHE*	pLSV1 based knock-out plasmid containing fragment GAEB for deletion of the *inlGHE* gene cluster by homologous recombination	(Raffelsbauer, 1997)
pLSVΔ*inlG*I	pLSV1 based knock-out plasmid containing fragment GAGB (not in-frame) for deletion of the *inlG* gene by homologous recombination	This study
pLSVΔ*inlG*II	pLSV1 based knock-out plasmid containing fragment GAGB (in-frame) for deletion of the *inlG* gene by homologous recombination	This study
pLSV*inlG*	pLSV1 based knock-in plasmid containing the *inlG* gene for reversion of the Δ*inlG* mutant by homologous recombination	This study
pLSVΔ*inlH*	pLSV1 based knock-out plasmid containing fragment HAHB for deletion of the *inlH* gene by homologous recombination	This study
pLSV*inlH*	pLSV1 based knock-in plasmid containing the *inlH* gene for reversion of the Δ*inlH* mutant by homologous recombination	This study
pLSVΔ*inlE*	pLSV1 based knock-out plasmid containing fragment EAEB for deletion of the *inlE* gene by homologous recombination	This study
pLSV*inlE*	pLSV1 based knock-in plasmid containing the *inlE* gene for reversion of the Δ*inlE* mutant by homologous recombination	This study
pUC18	Cloning vector, 2,686 bp, *lacZ$^+$*, ColE1-Origin, ampicillin resistance gene (ampicillin^R)	Pharmacia Biotech
pUC18Δ*inlG*I	pUC18 based plasmid containing fragment GAGB (not in-frame) for deletion of the *inlG* gene	This study
pUC18Δ*inlG*II	pUC18 based plasmid containing fragment GAGB (in-frame) for deletion of the *inlG* gene	This study
pUC18*inlG*	pUC18 based plasmid containing the *inlG* gene for reversion of the Δ*inlG* mutant	This study
pUC18Δ*inlH*	pUC18 based plasmid containing fragment HAHB for deletion of the *inlH* gene	This study
pUC18Δ*inlE*	pUC18 based plasmid containing fragment EAEB for deletion of the *inlE* gene	This study

Table 4.6: Plasmids (Continuation).

Plasmid	Properties	Source
pLSV16*gfp*	Shuttle vector containing the *gfp* cDNA encoding the green fluorescent protein GFP, fusion product of the vectors pUC18 and pBCE-1, ampicillin^R, tetracyclin^R	(Bubert *et al.*, 1999)
P*inlG-gfp*	pLSV16*gfp* based plasmid with *gfp* under control of the *inlG* promoter	This study
P*inlH-gfp*	pLSV16*gfp* based plasmid with *gfp* under control of the *inlH* promoter	This study
P*inlE-gfp*	pLSV16*gfp* based plasmid with *gfp* under control of the *inlE* promoter	This study
P*actA-gfp*	pLSV16*gfp* based plasmid with *gfp* under control of the *actA* promoter	(Bubert *et al.*, 1999)
P*inlA-gfp*	pLSV16*gfp* based plasmid with *gfp* under control of the *inlA* promoter	(Bubert *et al.*, 1999)
P*inlC-gfp*	pLSV16*gfp* based plasmid with *gfp* under control of the *inlC* promoter	(Bubert *et al.*, 1999)
P*hly-gfp*	pLSV16*gfp* based plasmid with *gfp* under control of the *hly* promoter	(Bubert *et al.*, 1999)
pLSV16	Shuttle vector without *gfp*, fusion product of pUC18 and pBCE-1, ampicillin^R, tetracyclin^R	(Bubert *et al.*, 1999)
pERL3	Shuttle vector, erythromycin^R, kanamycin^R	(Hartman *et al.*, 1990)
pERL3501	pERL3 derived plasmid containing the *prfA* gene from *L. monocytogenes* NCTC 7973	(Leimeister-W. *et al.*, 1990)
pERL3502	pERL3 derived plasmid containing the *prfA* and *plcA* genes from *L. monocytogenes* NCTC 7973	(Leimeister-W. *et al.*, 1990)

Table 4.7: Media for bacterial growth.

Name	Components	Quantity
BHI	BHI	38 g
	(brain-heart-infusion broth)	ad 1,000 ml H$_2$O$_{deion.}$
$2\times$ YT broth	Tryptone oder Peptone	16 g
	Yeast extract	10 g
	NaCl	10 g
		ad 1,000 ml H$_2$O$_{deion.}$

Table 4.8: Antibiotics.

Name	Conc. of stock solution		Final Conc.	Application
ampicillin	100 mg/ml	in $H_2O_{ster.}$	100 μg/ml	*E. coli*
erythromycin	100 mg/ml	in 70% EtOH	400 μg/ml	*E. coli*
erythromycin	5 mg/ml	in 70% EtOH	5 μg/ml	*Listeria* spp.
tetracycline	10 mg/ml	in 70% EtOH	4 or 7.5 μg/ml	*Listeria* spp.

Eukaryotic cell lines were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) according to standard procedures. Media and chemicals for cell culture were obtained from Life Technologies GmbH (Karlsruhe, Germany).

4.4 Enzymes, chemicals, plastic material, commercial kits and instruments

Enzymes used in this study were purchased from Amersham Pharmacia Biotech (Freiburg, Germany), Life Technologies GmbH (Karlsruhe, Germany), Promega (Madison, USA), New England Biolabs (Schwalbach, Germany), Q-Biogene (Heidelberg, Germany), Roche Diagnostics GmbH (Mannheim, Germany) and Stratagene (Heidelberg, Germany; La Jolla, USA, and Cedar Creek, USA). Used DNA molecular weight standards were supplied by Life Technologies GmbH (Karlsruhe, Germany) and Stratagene (La Jolla, USA). Sizes of single DNA bands are shown in Table 4.9. Chemicals were obtained from Aldrich Chemie (Steinheim, Germany), Carl Roth GmbH (Karlsruhe, Germany), Difco (Augsburg, Germany), Fluka (Neu-Ulm, Germany), Life Technologies GmbH (Karlsruhe, Germany), Merck (Darmstadt, Germany), Oxoid (Wesel, Germany), Riedel de Haen (Seelze, Germany), Roche Diagnostics GmbH (Mannheim, Germany), Serva (Heidelberg, Germany) and Sigma (Deisenhofen, Germany). Plastic material such as plates, tubes, flasks, dishes and pipettes were purchased from Becton Dickinson (Heidelberg, Germany), Greiner (Frickenhausen, Germany), Noras (Würzburg, Germany), Nunc (Wiesbaden, Germany) and Sarstedt (Nümbrecht, Germany). Commercial kits and instruments used are listed in Tables 4.10 and 4.11, respectively.

4.5 Oligonucleotides

Oligonucleotides used in this study, listed in Tables 4.12 and 4.13, were purchased from MWG-Biotech GmbH (Ebersbeg, Germany) or ARK Scientific GmbH Biosystems (Darmstadt, Germany). Nucleotide sequence is given in the $5' \rightarrow 3'$ direction. Restriction site, if any, is indicated.

Table 4.9: DNA molecular weight standards.

Name	1 kb Ladder (Life Technologies)		kb DNA Ladder (Stratagene)	
Size of band	$12,216$ bp	$1,635$ bp	$12,000$ bp	$1,000$ bp
	$11,198$ bp	$1,018$ bp	$10,000$ bp	750 bp
	$10,180$ bp	$516/506$ bp	$9,000$ bp	500 bp
	$9,162$ bp	394 bp	$8,000$ bp	250 bp
	$8,144$ bp	344 bp	$7,000$ bp	
	$7,126$ bp	298 bp	$6,000$ bp	
	$6,108$ bp	220 bp	$5,000$ bp	
	$5,090$ bp	200 bp	$4,000$ bp	
	$4,072$ bp	154 bp	$3,000$ bp	
	$3,054$ bp	142 bp	$2,000$ bp	
	$2,036$ bp		$1,500$ bp	

Table 4.10: Commercial kits used in this study.

Application	Name	Manufacturer
Purification of DNA fragments	GFX PCR DNA and gel band purification kit	Amersham Pharmacia Biotech (Freiburg, Germany)
	QIAquick PCR Purification Kit	QIAGEN (Hilden, Germany)
	NucleoTrap® Extraction Kit	Macherey-Nagel (Düren, Germany)
Isolation of Plasmid-DNA (Miniprep)	QIAprep Spin Plasmid Kit	QIAGEN (Hilden, Germany)
	Quantum Prep® Plasmid Miniprep Kit	BIO-RAD (München, Germany)
Isolation of Plasmid-DNA (Midiprep)	NucleoBond® PC 100 Kit	Macherey-Nagel (Düren, Germany)
Nucleotide sequencing	ABI PRISM™ Dye and dRhodamine Terminator Cycle Sequencing Ready Reaction Kits	Perkin Elmer (Weiterstadt, Germany)
Isolation of bacterial RNA	E.Z.N.A Bacterial RNA Kit	PEQLAB Biotechnologie GmbH (Erlangen, Germany)
Synthesis of first-strand cDNA	ProSTAR First-Strand RT-PCR Kit	Stratagene (Cedar Creek, USA)
Detection of apoptosis	Cell Death Detection ELISAPLUS Kit	Roche Diagnostics GmbH (Mannheim, Germany)

Table 4.11: Instruments used in this study.

Name	Manufacturer
Autoclaves	Webeco, Münchener Medizin Mechanik
Balances	Labor Alliance, Sartorius, Mettler
Centrifuges	Beckmann, Heraeus
Centrifuges for cell culture	Heraeus, Sorvall
Clean benches	Nuaire, Gelaire, Envirco
ELISA Reader	BioRad
Electrophoresis chambers	cti
Energy suppliers	Bio-Rad, Desaga
FACS Epics Elite ESP cell sorter	Coulter
FastPrep FP120 Shaker	BIO101-Savant Instruments
Fluorescence microscope	Leica
Fluorimeter	SPEX
Incubators	Heraeus, Salvis
Incubator for cell culture	Nunc Cellstar
Klett photometer	Summerson
Magnetic stirrer	Gerhardt
Micropipettes	Gilson, Eppendorf
Microwave	AEG
Photometers	Zeiss, Pharmacia Biotech
pH meter	WTW pH 523
Shakers	Infors AG
Sonifier	Branson Sonic
Speed VAC concentrator	Eppendorf
Stoves	Heraeus
Table centrifuges	Eppendorf, Heraeus, Hettich
Thermoblocks	Liebisch, Eppendorf
Thermocyclers	bio-med, Perkin Elmer, Techne Progene
UV photo apparatus	Cybertech CS1 Mitsubishi
UV light	Desaga
Vortex	Heidolph, Boskamp
Water bath	Memmert

Table 4.12: Oligonucleotides used in this study.

Name	Sequence ($5' \to 3'$)	Restriction site
lism51-1	ACGACAAGTCAAGGTGCGG	
lism51-2	GTAGCAGGTGCAATAGGCG	
lism51-3	CTAGTTTTAATGGTACCTGCTATTCTCGG	*Kpn*I
lism51-4	CTGTTCCACTGCAGGGAACCGTTG	*Pst*I
lism51-11	CCCATTTAGTTCCACCCG	
lism51-14	TCTCTATAAAGGCAAACTCC	
lisli-15	CAAGTCATGGGCTGCAGGCTCAGGCAGAGAG	*Pst*I
lism51-24	CTGCGTGATGCTCTCTGCCC	
lism51-27	GAGAGGGATTCTGCAGAAACGGTGG	*Pst*I
lisminv-1	CCACACCTTGGATCCTCGTTACATATAGG	*Bam*HI
lisminv-2	CGCAGCCAACTGCAGTTAATGTGATTTTCCC	*Pst*I
lisminv-4	ATAAGATTAGGGTACCTAGCAAGTGGCG	*Kpn*I
lisminv-5	GTTAGTTATACTTGGTACCAATCAGTCACTTTC	*Kpn*I
lisminv-6	CCTTGGCTTGTGGTACCATATAGGCTAAGCC	*Kpn*I
lisminv-7	CTACTTTATCTGCAGTTGGGACTGGAGTAAC	*Pst*I
lisminv-8	TTATCTATCCTGCAGTCCCATTTATTCCCG	*Pst*I
lisminv-9	CAATTTATCTGCAGTAATCGGTAACTTGATG	*Pst*I
lisminv-10	TAAGAAGGAGGTACCCTTTATAGAGAACGG	*Kpn*I
lisminv-11	GAAACTATACTGCAGCGGCGCAACGG	*Pst*I
delxy-2	CCCACAAGGATCCAATTCTGCAATCG	*Bam*HI
delxy-3	AACTAATTCCCGGGATAGCTGTCAC	*Psp*A1
delxy-4	CGGAATTGGCCCGGGTTTATTCCGC	*Psp*A1
delxy-5	GATTGGTTTATTTTGTTTGACG	
delxy-6	CGTTACTGGTACCAATTTCGCTTAC	*Kpn*I
delxy-6b	CGTTACTGGATCCAATTTCGCTTAC	*Bam*HI
delxy-7	TATGCCCTGCAGATGCCAAAACC	*Pst*I
delxy-8	ACATCTTTCGTGTAGAAGGG	
delxy-9	TTTATTCCGCAAAAAACGTC	
delseq-1	AACCAATTCTGCAATCGCTG	
inlX-1	CCAAGTTCCGTCTTCACCTC	
inlX-2	TTAGGAACGCTTTTCATCGG	
inlX-3	AGTTATCTGGTACCAATCTGTTTGCG	*Kpn*I
orfZ-1	AATCAATATTTACCAAGTCC	
orfZ-2	GTACTAAACTGCAGTAAAAGCAAG	*Pst*I
pGluco-1	AGTAAGTGCCTCCACAAGCG	
pGluco-2	GTAAGTGCCTGCAGAAGCGAAATGTCC	*Pst*I
inlY-1	CATTTGATACTGCAGGTAAATTAACGAC	*Pst*I
inlY-2	GCTTTGATAGGTACCTTTTTGAGTCG	*Kpn*I
Xspez-1	GTAAAACCGTTAAATCAGTC	
YC2spez-2	GGGAAGCAAACAAGTGTGAC	
PinlXu	GGTGAAGATCTAGATTAATAGGTAGAGTAC	*Xba*I
PinlXd	TTTATCGTCTCTAGATAATTTGACTGAG	*Xba*I
PinlYu	ACTAAACTTCTAGAAACAAGTGATGATTC	*Xba*I
PinlYd	ATAATCCCTCTAGATTTATTTAAATTAG	*Xba*I

Table 4.13: Oligonucleotides (Continuation).

Name	Sequence ($5' \rightarrow 3'$)	Restriction site
PinlZu	CGGGGTTTTCTAGATAGGAACAGCGACGC	*Xba*I
PinlZd	CTATCTCCTCTAGAAAGTAATTCTGC	*Xba*I
PinlZ	CCTTTATAAAGTAATTCTGC	
mutrevG-1	TTTACGCGAATTCCCACAAGAAACC	
delG-1	GCTTTTCATCCCGGGTGCAATACTAATC	
delH-1	TCATCGGAGGATCCATACTAATCTTACG	
delH-2	CCTCCACACCCGGGCTTGTCGTTAC	
delH-3	GATTTTTATCCCGGGTTTCTTGCTAGG	
delH-4	AGTGACTAGGATCCTTAATGCTGTTTTG	
delE-1	TAGGAACAGGGATCCTAATTCTCCGC	
delE-2	TTAATAGGGCCCGGGTGTTCAATCACC	
InlC-1b	AAACATCTCGGATCCTTGCTAACATATAAG	
InlC-2b	TTTGTCAAGAATTCATTAAGACTTAC	
InlC-3	ATAGCAGACAACAAGGAAGG	
InlC-4	ATAGTTTCTTCATCTCTTGG	
delCseq-2	CTTCATCTGTATAAACTGGC	
Xvorw-6	CATTTGTAGGATGGTATGAT	
inlYseq-1	CCCAAGTAATTCAGGAGGC	
inlYseq-2	AGAATTACTTTATAAAGGAG	
inlZseq-3	CACTTACTTGAGTACTTCCG	
lism51-14	TCTCTATAAAGGCAAACTCC	
RTG7Bdown	AATGTTAACTGTGTCAATCC	
RTH5down	GACAATAAATAAGTTTGAAG	
RTE3Ddown	CAATAGATAAGTTGGGTAAT	
RTA2down	CTAGTTAAACCTGAAAGCGC	
RTB2down	AGTTAAACGTGAAAGAACCG	
RTC2down	TTACCATGCAAATCTAATAC	
SOD-C	GTTCCCAAACATCTAAGCC	
RTG1up	ACAGAGAAAAACCTCAGTAC	
RTH3up	AATGCAATTAAAATAGCGGC	
RTE3Aup	ATAATGTAAAAACACTGCTC	
RTA1up	ATATTAGTATTTGGCAGCGG	
RTB1up	TGTTTAATCTCAGGTTTAGC	
RTC1up	TGTTAATTGTAGGTCTGTGC	
SOD-N	CTTATGATGCTTTGGAGCCG	
inlF-2	GCAGTTGGGGTACCACCAAATAGTGG	*Kpn*I
inlF-6	GCCTGTTTCTGCAGCATACCAGCCATC	*Pst*I
inlFinv-1	TAAGTTGGCTGCAGGACTTATGTCAG	*Pst*I
inlFinv-2	ATGTAGAAGGTACCACAAGTGAAGTAG	*Kpn*I
MonoA	CAAACTGCTAACACAGCTACT	
MonoB	GCACTTGAATTGCTGTTATTG	
gfp75	CAAGAATTGGGACAA	
gfp230	TGCTGTTTCATATGATCTGG	
Reverse	CAGGAAACAGCTATGAC	
Universal	GTAAAACGACGGCCAGT	

Methods

This Chapter describes methods and strategies used in the present study. In particular, experiments concerning the molecular cloning of the *inlGHE* gene cluster, construction of *L. monocytogenes* internalin deletion mutants, transcriptional studies using a GFP based expression system and a semi-quantitative RT-PCR technique, cellular invasion and intracellular growth assays, fluorescence microscopy, detection of cell death, and *in vivo* studies using the mouse model are described in detail. For more general genetic, biochemical and cell biological methods see the given references or standard protocols.

5.1 Genetic methods

5.1.1 Preparation of DNA

Chromosomal DNA was isolated from *Listeria* spp. as described in detail in Raffelsbauer (1997). Bacteria harvested from 14 ml overnight culture were treated with 20 mg lysozyme (Merck) in a 20% sucrose buffer and lysed with 1% SDS. The chromosomal DNA was extracted to purity with phenol/chloroform and precipitated with ethanol. In some cases, chromosomal DNA was isolated from *L. monocytogenes* by using 0.5 ml DNAzol Reagent (Life Technologies) as recommended by the manufacturer after treatment of bacteria harvested from 3 ml overnight culture with 1 mg lysozyme for 30 min at 37°C.

L. monocytogenes DNA was prepared for inverse PCRs using a method adapted from Ochman *et al*. (1990) as described in Raffelsbauer (1997). Chromosomal DNA was completely digested with the restriction enzymes *Alu*I, *Dra*I, *Eco*RI, *Eco*RV, *Rsa*I, *Sau*3A or *Taq*I. The digested DNA was then religated with T4 DNA ligase (Life Technologies) to create circular DNA molecules, which were used in inverse PCRs as described in 5.1.2.

For screening of *L. monocytogenes* strains by PCR, genomic and plasmid DNA was isolated as follows: bacteria were scraped from agar plates and resuspended in 50 μl polymerase buffer (Q-Biogene). Approx. 0.2 mg lysozyme (Merck) was added and bacterial suspensions were incubated 15 min at 37°C. After addition of 15 μg proteinase K (Merck), samples were incubated for further 10 to 20 min at 55°C and then for 10 min at 105°C. Cell debris were pelleted by centrifugation.

For PCR screening, plasmid DNA was isolated from *Escherichia coli* by incubating bacteria in 50 μl dH$_2$O for 10 min at 110°C. For restriction and nucleotide sequence analysis, *E. coli* plasmid DNA was isolated by using the commercial kits QIAprep spin plasmid kit (QIAGEN), the Quantum Prep Plasmid Miniprep kit (Bio-Rad Laboratories GmbH, München, Germany) or the NucleoBond PC 100 kit (Macherey-Nagel) as recom-

mended by the manufacturers.

5.1.2   Polymerase Chain Reaction (PCR)

Chromosomal and plasmid DNA of *L. monocytogenes* was amplified by Polymerase
Chain Reaction (PCR) according to standard procedures (Saiki *et al.*, 1988; Ochman *et al.*,
1990) using *Taq* DNA polymerase (Q-Biogene, Heidelberg, Germany), the Expand High
Fidelity PCR system enzyme mix (Roche Diagnostics GmbH, Mannheim, Germany) or
Deep Vent DNA polymerase (New England Biolabs, Schwalbach, Germany). Reactions
were performed as described in detail in Raffelsbauer (1997). A Standard PCR and pro-
gram used with the *Taq* DNA polymerase are shown in Tables 5.1 and 5.2, respectively.
Oligonucleotides used in this study to amplify DNA by PCR are listed in Chap. Mate-
rial, Tables 4.12 and 4.13. For cloning purposes, restriction sites were inserted in some
oligonucleotides.

Component & Concentration	Volume
$dH_2O_{ster.}$	$77, 5~\mu l$
$10\times$ *Taq* Polymerase Buffer	$10, 0~\mu l$
25 mM $MgCl_2$	$6, 5~\mu l$
20 mM dNTPs	$1, 0~\mu l$
Primer a ($1~\mu g/\mu l$)	$1, 0~\mu l$
Primer b ($1~\mu g/\mu l$)	$1, 0~\mu l$
chromosomal DNA ($0, 2 - 0, 4~\mu g/\mu l$)	$2, 0~\mu l$
Taq DNA Polymerase ($5~U/\mu l$)	$1, 0~\mu l$
	$100, 0~\mu l$

Table 5.1: Standard PCR with *Taq* DNA polymerase

Step	Event	Duration	Temperature	Cycles
1.	First step delay	3 min	94°C	
2.	Denaturation of dsDNA	1 min	94°C	
3.	Annealing of primers	1 min	55°C	$30\times$
4.	Elongation	1 min	72°C	
5.	Last step delay	3 min	72°C	

Table 5.2: Standard program for direct PCRs.

To amplify DNA fragments with unknown sequence located up- and downstream from
DNA regions whose sequences were available, inverse PCRs were performed as previ-
ously described (Raffelsbauer, 1997; Ochman *et al.*, 1990). Tables 5.3 and 5.4 show a
standard reaction and program for inverse PCRs.

Component & Concentration	Volume
$dH_2O_{ster.}$	$68,5\ \mu l$
$10\times$ *Taq* Polymerase Buffer	$10,0\ \mu l$
25 mM $MgCl_2$	$6,5\ \mu l$
20 mM dNTPs	$1,0\ \mu l$
Primer a (1 $\mu g/\mu l$)	$1,5\ \mu l$
Primer b (1 $\mu g/\mu l$)	$1,5\ \mu l$
religated DNA	$10,0\ \mu l$
Taq DNA Polymerase (5 U/μl)	$1,0\ \mu l$
	$100,0\ \mu l$

Table 5.3: Standard inverse PCR

Step	Event	Duration	Temperature	Cycles
1.	First step delay	3 min	94°C	
2.	Denaturation of dsDNA	55 sec	94°C	
3.	Annealing of primers	1 min	57°C	44×
4.	Elongation	1 min 30 sec	72°C	
5.	Last step delay	3 min	72°C	

Table 5.4: Standard program for inverse PCRs.

5.1.3 General techniques of molecular cloning

Molecular cloning was performed as described previously in Raffelsbauer (1997) or in standard protocols (Sambrook *et al.*, 1989) or as recommended by the manufacturers of the enzymes and commercial kits used. PCR products were purified directly or after electrophoretic separation on agarose gels using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany), the GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech) or the NucleoTrap extraction kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturers. After purification, the obtained PCR products were digested with appropriate restriction enzymes and then ligated into the chosen cloning vector with T4 DNA ligase (Life Technologies). DNA restriction, treatment with alkaline phosphatase and ligation were performed according to standard procedures as described in detail in Raffelsbauer (1997).

E. coli DH5α was used as cloning host for construction, analysis and amplification of all plasmids used in this work. Plasmids were introduced into *E. coli* by transformation after $MgSO_4$ treatment and into *L. monocytogenes* by electroporation using a method described previously by Alexander *et al.* (1990) and optimized by Bambach (1993). The nucleotide sequence of obtained PCR products was determined by sequencing either the purified products directly or after cloning into the vector using the ABI PRISM™ dye or the dRhodamine terminator cycle sequencing ready reaction kits (Perkin Elmer, Weiterstadt, Germany) according to protocols supplied by the manufacturer.

5.2   Construction of *L. monocytogenes* internalin deletion mutants

5.2.1   Construction of *inlGHE* deletion mutants

The *L. monocytogenes* mutant strains listed in Table 4.2 carrying an in-frame deletion in the *inlGHE* gene locus were constructed from the parental strains *L. monocytogenes* EGD wild type, Δ*inlA*, Δ*inlB*, Δ*inlC*, Δ*inlA/B* and Δ*inlB/C* by double cross-over based on a procedure described in Raffelsbauer (1997) using the temperature-sensitive shuttle vector pLSV1 (Wuenscher *et al.*, 1991). The applied strategy is represented in Fig. 2.10. In brief, DNA fragments GA (291 bp) and EB (364 bp), homologous to the 5' regions of the *inlG* and *dapE* genes respectively, were amplified from *L. monocytogenes* wild type chromosomal DNA by PCR using the primer pairs delxy-2/delxy-3 and delxy-4/delxy-6b, respectively. After restriction using the PCR-inserted *Psp*A1 sites, fragments GA and EB were sticky-ligated to yield fragment GAEB (632 bp). The precipitated ligation reaction was used as template DNA in a new PCR with the external primers delxy-2/delxy-6b. The thus amplified fragment GAEB was cloned into pUC18 via the inserted *Bam*HI restriction sites, and then recloned into pLSV1 via *Bam*HI. This shuttle vector carries an erythromycin resistance gene (Em^R), a Gram-negative and a temperature-sensitive Gram-positive origin of replication (Ori$_{ts}$). The resulting knock-out vector pLSVΔ*inlGHE* was screened by restriction analysis and nucleotide sequencing before being introduced into the recipient *L. monocytogenes* strains by electroporation as previously described (Bambach, 1993; Alexander *et al.*, 1990). Screening of erythromycin resistant clones was performed by PCR using the primer pair delxy-8/ORFZ-1. Integration of the vector into the chromosome by homologous recombination was induced by incubating bacteria at 42°C in the presence of erythromycin (5 μg/ml). Obtained clones were screened by PCR using the primers pGluco-1 and ORFZ-1, which selected integration at the 5' side (via fragment GA). The second cross-over (via fragment EB) leading to the excision of the vector and wild type sequence from the chromosome was accomplished by subculturing a selected integration strain 10 to 20 times in BHI at 30°C in the absence of erythromycin. Appropriate dilutions of this strain were plated onto BHI agar and clones were picked onto BHI plates with and without erythromycin. Sensitive clones were analyzed by PCR screening for the deletion of *inlGHE* using the primers PinlXu and delxy-7, which generate a shortened PCR product of 728 bp instead of the 5.6-kb wild type sequence. Obtained PCR products were cloned into pUC18 and sequenced. The correct in-frame deletions on the chromosome were verified by nucleotide sequence analysis of the PCR products. By the deletion of *inlGHE*, the first 56 bp of the coding region of *inlG* were fused in-frame to the last 34 bp of *inlE*. The resulting *inlGE* fusion gene is preceeded by the *inlG* promoter and encodes a short peptide of 29 amino acids.

All remaining mutant strains were obtained by using a similar strategy applied for the construction of the Δ*inlGHE* deletion mutants described above. The mutant strains *L. monocytogenes* Δ*inlA/C* and *L. monocytogenes* Δ*inlA/C/GHE* were constructed by deleting the *inlC* gene from the *L. monocytogenes* mutants Δ*inlA* and Δ*inlA/GHE*, respectively. A 243-bp long truncated *inlC* gene carried by the strain *L. monocytogenes*

Δ*inlC* (Engelbrecht *et al.*, 1996) was amplified by PCR using the primer pairs InlC-1b/InlC-2b and cloned into pLSV1 via the restriction sites *Bam*HI and *Eco*RI, which were inserted in the primers. Correct cloning was confirmed by sequencing the insert of the resulting vector pLSVΔ*inlC*. Selection of positive clones after electroporation and vector integration into the chromosome was achieved by PCR screening using the primer pairs InlC-1b/InlC-2b and InlC-3/delCseq-2, respectively. Erythromycin sensitive deletion mutants were isolated by PCR screening with the primers InlC-3 and InlC-4, which generate a 578-bp long product containing the truncated *inlC* gene (243 bp). Sequencing of the obtained PCR products revealed the same nucleotide sequence as in the *L. monocytogenes* Δ*inlC* mutant strain.

5.2.2 Construction of *inlG*, *inlH* and *inlE* single mutants and revertants

The single deletion mutants *L. monocytogenes* Δ*inlG*, Δ*inlH* and Δ*inlE* were constructed by deleting the corresponding genes from the strain *L. monocytogenes* EGD wild type. To generate homology fragments for deletion of *inlG* (GA and GB), *inlH* (HA and HB) and *inlE* (EA and EB), following primer pairs were used: GA (mutrevG-1/delxy-3), GB (delG-1/lisminv-1), HA (delH-1/delH-2), HB (delH-3/delH-4), EA (delE-1/delE-2) and EB (delxy-4/delxy-6b). These fragments were cut with *Psp*A1 and ligated to yield the fragments GAGB, HAHB and EAEB, which were amplified in subsequent PCRs using the corresponding external primers. The obtained fragments were cloned into pLSV1 via *Eco*RI (GAGB) or *Bam*HI (HAHB and EAEB) restriction sites, resulting in the knock-out plasmids pLSVΔ*inlG*, pLSVΔ*inlH* and pLSVΔ*inlE*, respectively.

To construct pLSV1-based knock-in vectors in order to complement the single mutants *L. monocytogenes* Δ*inlG*, Δ*inlH* and Δ*inlE* with a copy of the corresponding gene integrated into the chromosome and thus revert these mutants to the wild type genotype, the genes *inlG*, *inlH* and *inlE* were amplified from *L. monocytogenes* wild type chromosomal DNA using the same external primers used for the construction of the deletion strains, i. e. mutrevG-1/lisminv-1 for *inlG*, delH-1/delH-4 for *inlH* and delE-1/delxy-6b for *inlE*. The obtained products were cloned into pLSV1 via *Eco*RI (*inlG*) or *Bam*HI (*inlH* and *inlE*) restriction sites, yielding the knock-in plasmids pLSV*inlG*, pLSV*inlH* and pLSV*inlE*. The correct construction of all mutagenesis vectors described above was confirmed by nucleotide sequence analysis. Selection of positive clones after electroporation, chromosomal integration of the vector and allelic exchange on the chromosome was performed using erythromycin as selection marker and by PCR screening with appropriate primers. The correct in-frame deletions and reversions were confirmed by nucleotide sequence analysis of the obtained PCR products. The DNA sequences of the constructed *inl* deletion and reversion mutants are listed in App. B.2.

5.3 Transcriptional studies

5.3.1 Determination of promoter activity based on a GFP expression system

A schematic representation of the construction of the GFP expression plasmids P*inlG-gfp*, P*inlH-gfp* and P*inlE-gfp* is shown in Fig. 2.7. Briefly, the putative promoter regions of the genes *inlG*, *inlH* and *inlE* were amplified by PCR using the primer pairs PinlXu/PinlXd, PinlYu/PinlYd and PinlZu/PinlZd, respectively. The obtained PCR products were cleaved with the restriction enzyme *Xba*I and cloned into the shuttle vector pLSV16-*gfp* (Bubert *et al.*, 1999) via the *Xba*I restriction site. The plasmid pLSV16-*gfp* is a fusion product of pUC18 and pBC16-1 which carries in addition to the ampicillin and tetracycline resistance genes the gene encoding the green fluorescent protein GFP (Cormack *et al.*, 1996), flanked by a ribosome binding site (AGGAG) at the 5' end (Bubert *et al.*, 1999). The thus constructed plasmids P*inlG-gfp*, P*inlH-gfp* and P*inlE-gfp* were amplified in *E. coli* DH5α cells. Nucleotide sequence analysis of the inserts of these plasmids revealed wild type nucleotide sequences. The plasmids were then introduced into *L. monocytogenes* EGD wild type strain and into an isogenic *prfA* deletion mutant (Böckmann *et al.*, 1996) by electroporation as previously described (Bambach, 1993; Alexander *et al.*, 1990). EGD wild type strains harboring GFP constructs were complemented with additional copies of PrfA by introducing the wild type *prfA* gene cloned into the vector pERL3 50-2 (Leimeister-Wächter *et al.*, 1990). Obtained clones were selected with corresponding antibiotics and screened by PCR. Primer pairs used to detect plasmids were delseq-1/gfp75 (P*inlG-gfp*), inlX-2/gfp75 (P*inlH-gfp*), PinlZu/gfp75 (P*inlE-gfp*) and PrfA1/PrfA2 (pERL3 50-2). To detect chromosomal wild type or mutant *prfA* primers CO2 and prs3 were used. Bacterial strains containing GFP based expression vectors used in this work are listed in Table 4.3.

The promoter activities based on GFP expression were determined as follows: overnight cultures were diluted in fresh BHI medium and grown at 37°C in the presence of tetracycline (4 μg/ml) to an optical density of 1.2 at 550 nm. Two ml samples of each culture were centrifuged (12,000 × g, 1 min, room temperature), the cells were washed twice with PBS, resuspended in 2 ml PBS and then diluted 1:100 in PBS. Fluorescence was measured in a SPEX fluorimeter with a fixed excitation wavelength of 480 nm and a fixed emission wavelength of 508 nm using the strains *L. monocytogenes* EGD wild type, Δ*prfA* or wild type complemented with *prfA* as blank values. For the MEM shift, bacteria were grown as described above, washed with PBS and resuspended in 2 ml of prewarmed MEM. Bacteria were then incubated at 37°C in 5% CO_2 for 1 h. After this incubation, bacterial cells were centrifuged, washed and diluted 1:100 in PBS as described above.

5.3.2 Isolation of *L. monocytogenes* RNA and semi-quantitative RT-PCRs

For transcriptional studies of the genes *inlG*, *inlH* and *inlE*, RNA was isolated basically using a method developed by Oelmüller *et al.* (1990) and described in Altrock (1997). To study transcription of the genes *inlA*, *inlB* and *inlC*, *L. monocytogenes* cells were lysed us-

ing a fast method recently described by Dietrich *et al.* (2000) and RNA was isolated using the E.Z.N.A. Bacterial RNA Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to instructions of the supplier with following modifications: *L. monocytogenes* wild type and internalin mutant strains were grown to an optical density of 1.0 at 600 nm. 3 ml of each culture were centrifuged and bacteria were stored at $-80°$C for 2 h. Bacterial pellets were resuspended in 200 μl of BRL buffer (PEQLAB). 1 ml of lysis buffer TRK (PEQLAB) containing 2% β-mercaptoethanol was added. Cell suspensions were transferred to FastRNA BLUE Tubes (BIO 101 Inc., Carlsbad, CA, USA) and processed for 2 × 45 sec at speed 6.5 in a FastPrep FP120 Shaker (BIO 101–Savant Instruments, Holbrook, NY, USA). The tubes were centrifuged for 1 min at 8,000 × *g* and 4°C. Supernatants were transferred to Eppendorf tubes, 0.8 vol of ethanol was added and solutions were applied to a HiBind column (PEQLAB). The RNA was bound to the column, washed, dried and eluted in 2 × 100 μl RNase-free H$_2$O as recommend by the manufacturer. RNA aliquots were stored at $-80°$C. To analyze transcription of the genes *inlA*, *inlB* and *inlC* along the growth curve, *L. monocytogenes* strains wild type and $\Delta inlGHE$ were grown to optical densities of 0.5, 1.0 and 1.4 at 550 nm. At each time point appropriate volumes of the bacterial cultures corresponding to 2 × 10^9 cells were harvested by centrifugation and RNA was isolated applying the above described method. To remove remaining DNA, RNA aliquots were treated with RNase-free DNase (Amersham Pharmacia Biotech) and the complete DNA digestion was confirmed by PCR using samples of the RNA aliquots prior to the reverse transcription as template. The RNA concentration of each aliquot was determined by measuring the optical density at 260 nm in three independent samples and calculating the mean. Reverse transcription (RT) was performed with the ProSTAR First-Strand RT-PCR Kit (Stratagene, Cedar Creek, TX, USA) according to the instructions of the supplier using the Moloney Murine Leukemia Virus reverse transcriptase and 5 μg total RNA as template (except RT of the *inlG*, *inlH* and *inlE* genes, for which 10 μg total RNA were used). For synthesis of 1st-strand cDNA the oligonucleotides RTG7Bdown, RTH5down, RTE3Ddown, RTA2down, RTB2down, RTC2down and SOD-C were used (for sequence see Table 4.13). For subsequent amplification of the cDNA by PCR, the oligonucleotides RTG1up, RTH3up, RTE3Aup, RTA1up, RTB1up, RTC1up and SOD-N and the aforementioned primers were used. Oligonucleotides specific for the *inlG*, *inlH*, *inlE*, *inlA*, *inlB* and *inlC* genes were designed after nucleotide sequence analysis using programs of the HUSAR resource (German Cancer Research Center, Heidelberg, Germany). The specificity of the designed primers was verified in control PCRs using chromosomal DNA as template.

5.4 Methods for bacteria and cell culture

5.4.1 Media and growth conditions of bacterial strains

Bacterial strains and plasmids used in this study are listed in Tables 4.1 to 4.6. *Listeria* spp. strains were grown aerobically in brain-heart-infusion broth (BHI; Difco Laboratories, Detroit Mich. USA; Life Technologies, Karlsruhe, Germany; Scharlau Microbiology, Barcelona, Spain) at 37°C, except strains containing pLSV1 (Wuenscher *et al.*, 1991)

vector derivatives, which were grown at either 30°C or 42°C in the presence of 5 μg/ml erythromycin. For the growth of *L. monocytogenes* strains containing pLSV16*gfp* (Bubert *et al.*, 1999) derived plasmids, tetracycline was added to a final concentration of 4 μg/ml to liquid media or 7.5 μg/ml to solid media. *L. monocytogenes* strains containing the vector pERL350-2 (Leimeister-Wächter *et al.*, 1990) were incubated in the presence of 5 μg/ml erythromycin. *Escherichia coli* strains were grown in 2× YT broth at 37°C. For strains containing pUC18 (Amersham Pharmacia Biotech, Freiburg, Germany) or pLSV16*gfp*, the media were supplemented with 100 μg/ml ampicillin. *E. coli* strains containing pERL350-2 or pLSV1 vector derivatives were grown in the presence of 400 μg/ml erythromycin. To keep the bacterial strains, overnight cultures were diluted 1:1 in a solution containing 50% glycerol and 2.9% NaCl and stored at −80°C. To prepare bacteria aliquots for infection assays and to isolate RNA overnight cultures of *L. monocytogenes* strains were diluted 1:25 in fresh BHI and grown to the desired optical densities. The shift to minimum essential medium (MEM; Life Technologies, Paisley, Scotland) was performed as described in section 5.3.1.

5.4.2   Cell culture, cellular invasion and intracellular growth assays

All cells used in this study were cultivated at 37°C in a humid atmosphere of 5 % CO_2 according to standard methods. Cells of the cell lines Caco-2, J774, P388 and TIB73 were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (FCS; Life Technologies). For the culture of HepG-2 cells, a complete HepG-2 medium was used. Before and after infection assays, cells were washed with 1× phosphate-buffered saline (PBS) complemented with 1× $CaCl_2$ and 1× $MgCl_2$. Bacteria used for infection were grown to the mid-log phase (180 Klett units), harvested by centrifugation, washed twice with PBS and then stored in aliquots in PBS with 20% glycerol (v/v) at −80°C. The number of bacteria (CFU) per ml was determined by plating on BHI plates serial dilutions of the bacteria aliquots in PBS. For cellular adhesion, invasion and intracellular growth assays, cells were split 48 h prior to infection and seeded into 60 mm-diameter tissue culture plates. The cell densities immediately prior to the assays were approximately 10^6 cells per plate (semi-confluent monolayer) To reach the desired multiplicity of infection (MOI) appropriate volumes of the bacteria aliquots were diluted in RPMI 1640 medium and the bacterial suspensions (2 ml per plate) were added to each monolayer. Cells were infected in FCS-free medium for 1 h (J774 and P388 cells for 45 min) at variable MOIs of 10 (for J774 and P388 cells), 20 (for Caco-2 and HepG-2 cells) and 30 (for TIB73 cells) bacteria per cell. After infection the monolayers were washed three times with PBS and overlaid with complete media containing 50 μg/ml gentamicin (Serva, Heidelberg, Germany) to kill extracellular bacteria. After 1 h of incubation monolayers were again washed with PBS and cells were lysed by addition of ice-cold distilled water and sonication for 3 × 1 sec with a Branson sonifier. The number of intracellular bacteria was determined by plating appropriate serial dilutions of the cell lysates on BHI plates.

5.4.3 Quantification of GFP fluorescence of intracellular bacteria

Caco-2 and J774 cells were seeded 48 h prior to infection in 60 mm-diameter tissue culture plates, washed with PBS, and infected with listeriae at a MOI of 50 (Caco-2 cells) or 10 (J774 cells) bacteria per cell in FCS-free RPMI 1640 medium. Bacteria used for infection were grown to the mid-log phase (180 Klett units), washed with PBS, and stored in PBS with 15% glycerol at $-80°$C. The numbers of CFU per ml were determined by 10-fold serial dilutions of the bacteria in PBS and plating onto BHI agar plates containing 7.5 μg/ml tetracycline. For infection, frozen aliquots were thawed and applied to the cultured mammalian cells. After 60 min (Caco-2 cells) or 45 min (J774 cells) incubation to allow optimal uptake of the bacteria, the plates were washed three times with PBS and overlaid with supplemented RPMI medium containing 25 μg/ml gentamicin to kill extracellular bacteria and to prevent reinfection. To determine GFP-mediated fluorescence intensities of bacteria grown in mammalian cells at various time points of infection, the gentamicin containing RPMI medium was removed from the infected host cells. Cells were trypsinized and transferred into a plastic tube. GFP fluorescence of bacteria in live infected cells was measured by flow cytometry using an Epics Elite ESP cell sorter (Coulter, Krefeld, Germany) using the 488 nm line of an argon ion laser. Quantification was achieved by using the green light channel (525 / 10 nm bandpass filter), while the red light channel (630 / 20 nm bandpass filter) was used to exclude dead cells following staining with propidium iodide (PI, 2 μg/ml). A threshold of 0.1% was set to discriminate between GFP-positive and -negative cells using control cells infected with *L. monocytogenes* without carrying the *gfp* gene. Analyzing 20,000 cells per infection, the total fluorescence value was calculated by multiplying the number of fluorescent cells with the mean fluorescence of the GFP-positive/PI-negative cells. Then, the number of bacteria in the same sample volume as analyzed by flow cytometry (approx. 300 μl) was determined by lysing the mammalian cells and platting out appropriate dilutions of the cell lysates onto BHI agar plates containing 7.5 μg/ml tetracycline. The fluorescence intensity per bacterium was calculated from the total fluorescence value and the number of viable bacteria.

5.4.4 Fluorescence microscopic studies

The GFP expression vectors P*inlG-gfp*, P*inlH-gfp* and P*inlE-gfp* described in section 5.3.1 containing the putative promoter region of *inlG*, *inlH* and *inlE*, respectively fused to the gene encoding the green fluorescent protein (GFP; Cormack *et al.*, 1996) cloned into the shuttle vector pLSV16*gfp* (Bubert *et al.*, 1999) were introduced into the *L. monocytogenes* wild type and the $\Delta inlA/B$ mutant strains by electroporation (Bambach, 1993; Alexander *et al.*, 1990). Positive clones were selected with tetracycline (7.5 μg/ml) and screened by PCR using the primer pairs delseq-1/gfp230 (for P*inlG-gfp*), inlX-2/gfp230 (for P*inlH-gfp*) and delE-1/gfp230 (for P*inlE-gfp*). 24 h prior to infection HepG-2 cells were split and seeded into 24-well tissue culture plates at a density of 2×10^5 cells per well. Overnight cultures of both *L. monocytogenes* strains containing the GFP expression vectors were diluted 1:25 in fresh BHI supplemented with tetracycline and grown to an optical density of 180 Klett units. 2 ml samples of each culture were centrifuged, washed twice with PBS, resuspended in PBS and diluted in Minimal Essential Medium

(MEM; Life Technologies). An appropriate volume of the bacteria suspensions was given to each well (containing approx. 3×10^5 cells) to reach a multiplicity of infection of 20 bacteria per cell. Each strain was applied to three independent wells. The cultures were incubated for 1 h to allow the bacteria to invade the cells. After infection the cell monolayers were carefully washed twice with PBS, overlaid with MEM supplemented with 10 % FCS and incubated for further 5 h at 37°C. During this time the GFP fluorescence activity of the bacteria incubated with and without cells was monitored using a fluorescence microscope (Leica). Caco-2 cells were infected with *L. monocytogenes* wild type and Δ*inlGHE* strains transformed with the GFP expression plasmid P*actA-gfp* (Dietrich *et al*., 1998) in a MOI of 20 bacteria per cell. GFP-mediated fluorescence was monitored 18 h post-infection using a fluorescence microscope.

5.4.5   Detection of apoptosis in Caco-2 and J774 cells infected with *L. monocytogenes*

Caco-2 and J774 cells were seeded in 96-well tissue culture dishes at a density of 10^4 cells/well in a total volume of 200 μl of RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FCS, and incubated under 5 % CO_2 atmosphere at 37°C for 48 h. Cells were infected at a MOI of 20 (Caco-2) and 0.5 (J774) bacteria per cell for 1 h in FCS-free medium. After infection complete medium containing gentamicin (25 μg/ml) was added to the cultures to kill extracellular bacteria. Quantitative determination of histone-associated DNA fragments was performed by using the photometric enzyme-immunoassay Cell Death Detection ELISAPLUS (Roche Diagnostics) according to the instructions of the manufacturer.

5.5   Other methods

5.5.1   *In vivo* studies

C57BL/6 mice were kept under specific-pathogen-free conditions in isolators and fed autoclaved food and water ad libitum at the central animal facilities of the University Clinics of Ulm. In a given experiment mice were age and sex matched. Five mice per group were orally infected with 2×10^8 bacteria of *L. monocytogenes* EGD or *L. monocytogenes* Δ*inlGHE*. At day 1 and 5 post infection colony forming units (CFU) of infected mice were determined by plating serial dilutions of organ homogenates on tryptic soy agar (Life Technologies).

5.5.2   Computer sequence analysis

For computational analyses of nucleotide and peptide sequence data the program package Clone Manager (Scientific & Educational Software), the program package of the Genetics Computer Group (GCG) of the University of Wisconsin, USA, or the HUSAR resource provided by the German Cancer Research Center, Heidelberg, Germany, were used. The Clone Manager was applied to manage nucleotide sequences and to search for restriction

sites. To search for homologous sequences in the database the fasta program was used (GCG). Peptide sequences were deduced with the translate program (GCG). For sequence alignments and calculation of percent identity and similarity, the bestfit program (GCG) was used. Multiple sequence alignments were performed with the programs pileup and prettybox (HUSAR). To design oligonucleotides specific for the *inlA*, *inlB*, *inlC*, *inlG*, *inlH* and *inlE* genes, sequences were analyzed using the programs pileup and prettybox (HUSAR) or findpatterns (GCG).

Strain collection

Table A.1: *Listeria monocytogenes* strains.

No.	Strain	Characteristics	Reference
S1	EGD wild type[a]	serotype 1/2a	S. H. E. Kaufmann
S2	EGD	serotype 1/2a	
S3	NCTC 7973	serotype 1/2a	NCTC
S4	SLCC 2755	serotype 1/2b	SLCC
S5	NCTC 5348	serotype 1/2c	NCTC
S6	NCTC 5105	serotype 3a	NCTC
S7	SLCC 5543	serotype 3b	SLCC
S8	SLCC 2479	serotype 3c	SLCC
S9	L99	serotype 4a	T. Chakraborty
S10	SLCC 4013	serotype 4b	SLCC
S11	ATCC 19116	serotype 4c	ATCC
S12	ATCC 19117	serotype 4d	ATCC
S13	ATCC 19118	serotype 4e	ATCC
S14	EGD $\Delta inlGHE$	first *inlGHE* mutant	Raffelsbauer *et al.*, 1998
S15	EGD $\Delta prfA$	mutant A42	Böckmann *et al.*, 1996
S16	EGD WT $\times$ pERL3501	Em resistant	Leimeister-W. *et al.*, 1990
S17	EGD $\Delta prfA \times$ pERL3502	Em resistant	
S18	EGD $\Delta inlA$		Greiffenberg *et al.*, 1997
S19	EGD $\Delta inlC$		Engelbrecht *et al.*, 1996
S20	EGD $\Delta inlA/B$		Greiffenberg *et al.*, 1997
S21	EGD $\Delta inlB$		Greiffenberg *et al.*, 1997
S22	EGD $\Delta inlB/C$		Greiffenberg *et al.*, 1997
S23	EGD $\Delta inlA/GHE$	mutant A2	This study
S24	EGD $\Delta inlA/GHE$	mutant A3	This study
S25	EGD $\Delta inlC/GHE$	mutant C11	This study
S26	EGD $\Delta inlC/GHE$	mutant C12	This study
S27	EGD $\Delta inlA/GHE$	mutant A2	This study
S28	EGD $\Delta inlA/GHE$	mutant A3	This study
S29	EGD $\Delta inlC/GHE$	mutant C11	This study
S30	EGD $\Delta inlC/GHE$	mutant C12	This study
S31	EGD $\Delta inlA/B/GHE$	mutant AB12	This study
S32	EGD $\Delta inlA/B/GHE$	mutant AB14	This study
S33	EGD $\Delta inlB/C/GHE$	mutant BC16	This study
S34	EGD $\Delta inlA/B/GHE$	mutant AB12	This study
S35	EGD $\Delta inlA/B/GHE$	mutant AB14	This study
S36	EGD $\Delta inlB/C/GHE$	mutant BC16	This study

[a]Wild type strain used for construction of all *inl* deletion mutants.

Table A.2: *Listeria monocytogenes* strains (continuation).

No.	Strain	Characteristics	Reference
S37	EGD Δ*inlG*I [a]	mutant FG12	This study
S38	EGD Δ*inlG*I [a]	mutant FG2	This study
S39	EGD Δ*inlE*	mutant Δ*inlE* 2-1	This study
S40	EGD Δ*inlE*	mutant Δ*inlE* 6-4	This study
S41	EGD Δ*inlH*	mutant Δ*inlH* 3-2	This study
S42	EGD Δ*inlH*	mutant Δ*inlH* 3-4	This study
S43	EGD WT × pLSVΔ*inlH*	clone 5, Em resistant, 30°C	This study
S44	EGD scom[b] pLSVΔ*inlH*	clone 3, Em resistant, 42°C	This study
S45	EGD scom pLSVΔ*inlH*	clone 5, Em resistant, 42°C	This study
S46	EGD WT × pLSVΔ*inlE*	clone EP1-6, Em resistant, 30°C	This study
S47	EGD WT × pLSVΔ*inlE*	clone EP3-3, Em resistant, 30°C	This study
S48	EGD scom pLSVΔ*inlE*	clone 6, Em resistant, 42°C	This study
S49	EGD scom pLSVΔ*inlE*	clone 2, Em resistant, 42°C	This study
S50	EGD WT × pLSVΔ*inlG*II	clone 4, Em resistant, 30°C	This study
S51	EGD scom pLSVΔ*inlG*II	clone 4, Em resistant, 42°C	This study
S52	EGD scom pLSVΔ*inlG*II	clone 5, Em resistant, 42°C	This study
S53	EGD Δ*inlG*II [c]	mutant Δ*inlG* 4-3	This study
S54	EGD Δ*inlG*II [c]	mutant Δ*inlG* 5-3	This study
S55	EGD WT × pLSVΔ*inlGHE*	clone 1, Em resistant, 30°C	This study
S56	EGD WT × pLSVΔ*inlGHE*	clone 4, Em resistant, 30°C	This study
S57	EGD Δ*inlGHE*	new mutant Δ*inlGHE* 1-2/8	This study
S58	EGD Δ*inlGHE*	new mutant Δ*inlGHE* 4-3/33	This study
S59	EGD Δ*inlGHE*	new mutant Δ*inlGHE* 4-3/55	This study
S60	EGD *inlE*+ [d]	revertant E3-4/11	This study
S61	EGD *inlG*I+ [e]	revertant B4-4/3	This study
S62	EGD *inlG*II+ [f]	revertant C2-2/34	This study
S63	EGD *inlG*II+ [f]	revertant C2-2/38	This study
S64	EGD Δ*inlA*	obtained from M. Kuhn	Greiffenberg *et al.*, 1997
S65	EGD Δ*inlB*	obtained from M. Kuhn	Greiffenberg *et al.*, 1997
S66	EGD Δ*inlC*	obtained from M. Kuhn	Engelbrecht *et al.*, 1996
S67	EGD Δ*inlB/GHE*	mutant B4-4/6	This study
S68	EGD Δ*inlB/GHE*	mutant B4-6/11	This study
S69	EGD *inlE*+ [d]	revertant E4-1/1	This study
S70	EGD *inlE*+ [d]	revertant E4-2/4	This study
S71	EGD Δ*inlA/C*	mutant AC3-6	This study
S72	EGD Δ*inlA/C*	mutant AC4-7	This study
S73	EGD Δ*inlA/C*	mutant AC4-8	This study
S74	EGD Δ*inlA/C*	mutant AC4-10	This study
S75	EGD Δ*inlA/GHE/C*	mutant AGC3-5	This study
S76	EGD Δ*inlA/GHE/C*	mutant AGC4-14	This study
S77	EGD *inlH*+ [g]	revertant HD6-1/5	This study
S78	EGD *inlH*+ [g]	revertant HD6-5/0	This study
S79	EGD *inlH*+ [g]	revertant H17	This study
S80	EGD *inlH*+ [g]	revertant H19	This study

[a]Deletion of *inlG* not in frame.

[b]The abbreviation "scom" means single cross-over mutant.

[c]Deletion of *inlG* in frame.

[d]Strain *inlE*+ was constructed by complementing mutant strain Δ*inlE* (S40) with *inlE*.

[e]Strain *inlG*I+ was constructed by complementing mutant strain Δ*inlG*I (S38) with *inlG*.

[f]Strain *inlG*II+ was constructed by complementing mutant strain Δ*inlG*II (S54) with *inlG*.

[g]Strain *inlH*+ was constructed by complementing mutant strain Δ*inlH* (S41) with *inlH*.

Table A.3: *Listeria monocytogenes* strains (continuation).

No.	Strain	Characteristics	Reference
A2	EGD Δ*inlA* × pLSVΔ*inlGHE*	Em resistant, 30°C	This study
C2	EGD Δ*inlC* × pLSVΔ*inlGHE*	Em resistant, 30°C	This study
G2	EGD WT × pLSVΔ*inlG*I	Em resistant, 30°C	This study
AB1	EGD Δ*inlA/B* × pLSVΔ*inlGHE*	Em resistant, 30°C	This study
BC4	EGD Δ*inlB/C* × pLSVΔ*inlGHE*	Em resistant, 30°C	This study
SCA2	EGD Δ*inlA* scom pLSVΔ*inlGHE*	Em resistant, 42°C	This study
SCG1	EGD WT scom pLSVΔ*inlG*I	Em resistant, 42°C	This study
SCC3	EGD Δ*inlC* scom pLSVΔ*inlGHE*	Em resistant, 42°C	This study
SCAB2	EGD Δ*inlA/B* scom pLSVΔ*inlGHE*	Em resistant, 42°C	This study
SCBC2	EGD Δ*inlB/C* scom pLSVΔ*inlGHE*	Em resistant, 42°C	This study

Table A.4: *Listeria monocytogenes* strains containinig GFP expression plasmids.

No.	Strain	Characteristics	Reference
G1	EGD WT × P*inlG-gfp*	Tc resistant	This study
G2	EGD Δ*prfA* × P*inlG-gfp*	Tc resistant	This study
G3	EGD WT × pERL3502 × P*inlG-gfp*	Em and Tc resistant	This study
G4	EGD WT × P*inlH-gfp*	Tc resistant	This study
G5	EGD Δ*prfA* × P*inlH-gfp*	Tc resistant	This study
G6	EGD WT × pERL3502 × P*inlH-gfp*	Em and Tc resistant	This study
G7	EGD WT × P*inlE-gfp*	Tc resistant	This study
G8	EGD Δ*prfA* × P*inlE-gfp*	Tc resistant	This study
G9	EGD WT × pERL3502 × P*inlE-gfp*	Em and Tc resistant	This study
G10	EGD WT × P*actA-gfp*	Tc resistant	Bubert *et al.*, 1999
G12	EGD WT × pERL3502 × P*actA-gfp*	Em and Tc resistant	Bubert *et al.*, 1999
G13	EGD WT × P*inlA-gfp*	Tc resistant	Bubert *et al.*, 1999
G14	EGD Δ*prfA* × P*inlA-gfp*	Tc resistant	Bubert *et al.*, 1999
G15	EGD WT × pERL3502 × P*inlA-gfp*	Em and Tc resistant	Bubert *et al.*, 1999
G16	EGD WT × P*hly-gfp*	Tc resistant	Bubert *et al.*, 1999
G17	EGD Δ*prfA* × P*hly-gfp*	Tc resistant	Bubert *et al.*, 1999
G19	EGD WT × P*inlC-gfp*	Tc resistant	Bubert *et al.*, 1999
G20	EGD WT × pLSV16 without *gfp*	Tc resistant	This study
G21	EGD Δ*inlGHE* (S14) × pLSV16 without *gfp*	Tc resistant	This study
G22	EGD Δ*inlA/B* × P*inlG-gfp*	Tc resistant	This study
G23	EGD Δ*inlA/B* × P*inlH-gfp*	Tc resistant	This study
G24	EGD Δ*inlA/B* × P*inlE-gfp*	Tc resistant	This study

Table A.5: *E. coli* recombinant strains.

No.	Strain	Characteristics	Reference
C1	DH5α × pCYB2::*inlG* clone G0-1	Amp resistant	Impact system
C2	DH5α × pCYB2::*inlG* clone G0-9	Amp resistant	Impact system
C3	DH5α × pCYB2::*inlG* clone G1-18	Amp resistant	Impact system
C4	DH5α × pUC18Δ*inlG*II	Amp resistant	This study
C5	DH5α × pLSVΔ*inlG*II	Em resistant	This study
C6	DH5α × pUC18*inlG*	Amp resistant	This study
C7	DH5α × pLSV*inlG*	Em resistant	This study
C8	DH5α × pUC18Δ*inlH*	Amp resistant	This study
C9	DH5α × pLSVΔ*inlH*	Em resistant	This study
C10	DH5α × pUC18Δ*inlE*	Amp resistant	This study
C11	DH5α × pLSVΔ*inlE*	Em resistant	This study
C12	DH5α × pLSV*inlH* clone H2	Em resistant	This study
C13	DH5α × pLSV*inlH* clone H3	Em resistant	This study
C14	DH5α × pLSV*inlH* clone H13	Em resistant	This study
C15	DH5α × pLSV*inlE*	Em resistant	This study
G32	DH5α × P*inlG-gfp*	Amp and Tc resistant	This study
G33	DH5α × P*inlH-gfp*	Amp and Tc resistant	This study
G34	DH5α × P*inlE-gfp*	Amp and Tc resistant	This study
G35	DH5α × pUC18Δ*inlG*I	Amp resistant	This study
G36	DH5α × pLSVΔ*inlG*I	Em resistant	This study
G37	DH5α × pLSV1	Em resistant	Wünscher *et al.*, 1991
G38	DH5α × pERL3502	Em resistant	Leimeister-W. *et al.*, 1990

Nucleotide sequences

B.1 Nucleotide sequence of the *inlGHE* gene cluster and its flanking genes

RBS = putative ribosome binding site
start = start codon
stop = stop codon
* = end of the protein

```
  1 TCGACCTAGT AAGTGCCTCC ACAAGCGAAA TGTCCAAACG TTACGGCTTC ATCTACGTAG
      D   L    V   S   A    S   T   E    M   S   K    R   Y   G   F    I   Y   V

 61 ACCAAGATGA CTGGGGCAAA GGAACATTAG AACGCTCCCG CAAAGATTCA TTCTTCTGGT
      D   Q   D    D   W   G   K    G   T   L    E   R   S    R   K   D   S    F   F   W

                                                 stop
                                                 ascB
121 ATAAAAAGT AATTGAAACA AATGGTGAAG ATTTAGATTA ATAGGTAGAG TACAGCCCCG
      Y   K   K    V   I   E   T    N   G   E    D   L   D    *

181 CTTTGGCGGG GCTGTTTTTT TACGCTACTT CCCACAAGAA ACCAATTCTG CAATCGCTGA

241 ACGAAAAGT TCAATTAATT GTCACATCTT TCGTGTAGAA GGGTCTAATG CGAGAAAAAA

301 TGGCGGGCAT CTTCTGTTAT AATTATTCAT CAAAGACTAG TAATATTTTA CGCCAAGTCA

361 TCCAAGATAG TGCAGATCCA AGGTGTATTT AATGATGAAT TATCTACTCA GTCAAATTAT

             RBS                start
                                inlG
421 AAGGAGACGA TAAAATGAAA CAGAGAAAAA CCTCAGTACT ACATGTTTTA CTTGTAGTGA
                          M   K    Q   R   K    T   S   V    L   H   V   L    L   V   V

481 CAGCTATCTT GGGAATTAGT TTATGGGTAA ATGCAAGTCA TGGGATGAAA GCTCAGGCAG
      T   A   I    L   G   I   S    L   W   V    N   A   S    H   G   M   K    A   Q   A

541 AGAGTATTGC GCAACCAGCG CCAATTAACG AAATTTTCAC GGATCCAGCA TTAGCGGACG
      E   S   I    A   Q   P   A    P   I   N    E   I   F    T   D   P   A    L   A   D

601 AGGTGAAGAC GGAACTTGGA AAAACTAGTG TCACTGATGA AGTTACGCAA ACAGATTTGA
      E   V   K    T   E   L   G    K   T   S    V   T   D    E   V   T   Q    T   D   L

661 ATCAGATAAC TAAACTTGAA GCAGACGACA AAGGAATAAA TTCAATAGAG GGAATACAAT
      N   Q   I    T   K   L   E    A   D   D    K   G   I    N   S   I   E    G   I   Q

721 ATTTAACTAA TTTGAATATG TTGGGTGTAT CTTCCAATCA GATTACTAAT ATTACACCTC
```

```
          Y   L   T     N   L   N   M     L   G   V     S   S   N     Q   I   T   N     I   T   P
 781 TTGCCAATCT TACTAATTTA GATTCTTTAT ATTTAGGAGA TAATAAAATT AGTGATGTGA
          L   A   N     L   T   N   L     D   S   L     Y   L   G     D   N   K     I   S   D   V

 841 CGCCACTTTC AGGATTGACA CAGTTAACAT TCGTACAATT ATCTATCAAT CAAATAAAAG
          T   P   L     S   G   L   T     Q   L   T     F   V   Q     L   S   I   N     Q   I   K

 901 ATGTGACACC TCTTGCTAAT CTAACGAAAT TAAATTATTT AGATTTACGA GAAAATCAAA
          D   V   T     P   L   A   N     L   T   K     L   N   Y     L   D   L   R     E   N   Q

 961 TAAGTGATGC AAGTCCTTTA GTTAATATGA CTGATTTAAC GGTTTTACAT TTAGAAAAAC
          I   S   D     A   S   P   L     V   N   M     T   D   L     T   V   L   H     L   E   K

1021 AACAAATAAC AGCCGCGCCA GTCGTATATC AAACTAATTT AGTTGCACCA GATATTTTGA
          Q   Q   I     T   A   A   P     V   V   Y     Q   T   N     L   V   A   P     D   I   L

1081 AAAATGCTTA TGGTGAAGTA GTACCACCAA CAACGATTAG TAATAACGGA ACCTTTGCTA
          K   N   A     Y   G   E   V     V   P   P     T   T   I     S   N   N   G     T   F   A

1141 GTCCAAATAT CACTTGGAAC TTAGATAGTT TCACGAGTGA GGTTAGTTAT GATTTTAATC
          S   P   N     I   T   W   N     L   D   S     F   T   S     E   V   S   Y     D   F   N

1201 AAAAAATCAC ACTAGGTGAT AATGGGAAGG TAACTTTTGC AGGAACTGTT GTTCAACCGA
          Q   K   I     T   L   G   D     N   G   K     V   T   F     A   G   T   V     V   Q   P

1261 TAGTAGAAGC GCCCGTGAAT TACATTACTA CATTTGATGT GGATGGAACG ACGACGACAG
          I   V   E     A   P   V   N     Y   I   T     T   F   D     V   D   G   T     T   T   T

1321 AAAACGTGGT AGTGGATACA TTAATAACCG AACCTGCTGA ACCGACAAAA GAAGGTTATA
          E   N   V     V   V   D   T     L   I   T     E   P   A     E   P   T   K     E   G   Y

1381 CTTTTTCTGG TTGGTATGAT GCGGAAACTG GTGGGAATGA ATGGGATTTT GCAGTAGATA
          T   F   S     G   W   Y   D     A   E   T     G   G   N     E   W   D   F     A   V   D

1441 AAATGCCGGC TACGAATATG ACACTTTACG CACAGTTCAC GATAAACAGC TACACAGCGA
          K   M   P     A   T   N   M     T   L   Y     A   Q   F     T   I   N   S     Y   T   A

1501 CATTTGATGT GGATGGTGAA ACAACTAATC AAAAAGTAGA TTACCAAGCT CTGCTACAAG
          T   F   D     V   D   G   E     T   T   N     Q   K   V     D   Y   Q   A     L   L   Q

1561 AACCGACTGC TCCGACGAAA GATGGCTACA CATTTGTAGG ATGGTATGAT GCAAAAACGG
          E   P   T     A   P   T   K     D   G   Y     T   F   V     G   W   Y   D     A   K   T

1621 GTGGAACTGA ATGGGATTTC GCAACTAGCA AAATGCCAAC TAGTGATATA ACTTTATATG
          G   G   T     E   W   D   F     A   T   S     K   M   P     T   S   D   I     T   L   Y

1681 CTAGATTTAC TAAAAATCCT AGCTCAGACA ATTCTCAAAC AGCTCCCGGA AAAGATGATA
          A   R   F     T   K   N   P     S   S   D     N   S   Q     T   A   P   G     K   D   D

1741 AAAACGACAA AGATAAACTA ACAATTAAAG CTAACGACAG CGCAGATGCG ACGAGTACTA
          K   N   D     K   D   K   L     T   I   K     A   N   D     S   A   D   A     T   S   T

1801 AACTTCCAAA AACAAGTGAT GATTCAAGTA TGATTCCTAC TATTTTAGGA ACGCTTTTCA
          K   L   P     K   T   S   D     D   S   S     M   I   P     T   I   L   G     T   L   F

                                                                        stop
                                                                        inlG
                                                                        ⏜
1861 TCGGAGGTGC AATACTAATC TTACGAAAAA AAACTACTAA CATTTAAGAT AAAGTAGATT
          I   G   G     A   I   L   I     L   R   K     K   T   T     N   I   *
```

```
1921 TGGTTTCTAT TTTCAGTAGA GACCAAATTT TTTTGTTAAT TTGGTCTAAA AAAGGGTATC
```

```
                                                                    RBS
1981 TATTATTAAT GACTTATTTA GAAGAATAAT TAGTGAATCT AATTTAAATA AAAGGAGAGG
```

```
          start
          inlH
2041 GATTATGAAA AAACGGTGGA ATTCAGTATT CAAACTAGTT TTAATGGTAA CTGCTATTCT
          M  K    K   R   W    N  S  V    F  K  L  V    L  M  V    T  A  I
```

```
2101 CGGGCTTAGC CTATATGTAA CGACAAGCCA AGGTGTGGAG GTTCGGGCAG AGAGCATCAC
      L  G  L  S    L  Y  V    T  T  S    Q  G  V  E    V  R  A    E  S  I
```

```
2161 GCAGCCAACC GCAATTAATG TGATTTTCCC TGATCCAGCT CTTGCGAATG CAATTAAAAT
      T  Q  P  T    A  I  N    V  I  F    P  D  P  A    L  A  N    A  I  K
```

```
2221 AGCGGCTGGA AAATCTAATG TAACAGATAC TGTCACGCAA GCGGATTTAG ATGGAATAAC
      I  A  A  G    K  S  N    V  T  D    T  V  T  Q    A  D  L    D  G  I
```

```
2281 TACTTTATCA GCATTTGGGA CTGGAGTAAC AACGATAGAA GGAGTGCAGT ACTTAAATAA
      T  T  L  S    A  F  G    T  G  V    T  T  I  E    G  V  Q    Y  L  N
```

```
2341 TTTGATAGGG TTAGAACTTA AAGATAACCA AATAACTGAT TTAACTCCTC TTAAAAATTT
      N  L  I  G    L  E  L    K  D  N    Q  I  T  D    L  T  P    L  K  N
```

```
2401 AACGAAAATA ACAGAACTTG AATTATCTGG AAACCCGTTA AAAAATGTGA GCGCGATTGC
      L  T  K  I    T  E  L    E  L  S    G  N  P  L    K  N  V    S  A  I
```

```
2461 TGGGTTACAA AGCATAAAAA CGCTAGACCT AACTTCTACG CAAATTACAG ATGTGACCCC
      A  G  L  Q    S  I  K    T  L  D    L  T  S  T    Q  I  T    D  V  T
```

```
2521 ACTTGCAGGT CTTTCCAATT TGCAGGTATT ATATTTGGAT CTCAATCAAA TAACCAATAT
      P  L  A  G    L  S  N    L  Q  V    L  Y  L  D    L  N  Q    I  T  N
```

```
2581 AAGCCCGCTT GCAGGACTAA CTAATTTACA ATACTTATCA ATCGGAAATG CCCAAGTAAG
      I  S  P  L    A  G  L    T  N  L    Q  Y  L  S    I  G  N    A  Q  V
```

```
2641 TGATTTAACC CCACTTGCTA ATTTATCTAA ACTAACTACT TTAAAAGCTG ATGATAATAA
      S  D  L  T    P  L  A    N  L  S    K  L  T  T    L  K  A    D  D  N
```

```
2701 AATAAGTGAT ATTTCGCCAC TTGCTAGTTT ACCTAATCTT ATAGAAGTTC ATTTGAAAAA
      K  I  S  D    I  S  P    L  A  S    L  P  N  L    I  E  V    H  L  K
```

```
2761 TAATCAAATT AGTGATGTTA GCCCACTTGC GAATACTTCA AACTTATTTA TTGTCACTTT
      N  N  Q  I    S  D  V    S  P  L    A  N  T  S    N  L  F    I  V  T
```

```
2821 AACGAATCAA ACAATTACCA ACCAACCTGT GTTTTATCAA AATAATCTTG TCGTCCCTAA
      L  T  N  Q    T  I  T    N  Q  P    V  F  Y  Q    N  N  L    V  V  P
```

```
2881 TGTAGTAAAA GGTCCTTCTG GCGCGCCTAT TGCACCCGCT ACTATTAGTG ACAATGGAAC
      N  V  V  K    G  P  S    G  A  P    I  A  P  A    T  I  S    D  N  G
```

```
2941 ATACGCAAGC CCAAATTTAA CATGGAATTT AACTAGTTTT ATTAATAATG TTAGTTATAC
      T  Y  A  S    P  N  L    T  W  N    L  T  S  F    I  N  N    V  S  Y
```

```
3001 TTTTAACCAA TCAGTCACTT TCAAAAATAC AACAGTTCCG TTTAGTGGGA CAGTTACACA
      T  F  N  Q    S  V  T    F  K  N    T  T  V  P    F  S  G    T  V  T
```

```
3061 ACCATTAACA GAAGCTTACA CTGCGGTTTT TGACGTGGAC GGGAAGCAAA CAAGTGTGAC
      Q  P  L  T   E  A  Y   T  A  V   F  D  V  D   G  K  Q   T  S  V

3121 AGTCGGCGCG AATGAATTAA TTAAAGAACC AACGGCCCCG ACGAAAGAAG GTTACACTTT
      T  V  G  A   N  E  L   I  K  E   P  T  A  P   T  K  E   G  Y  T

3181 CACAGGCTGG TATGATGCGA AAACTGGCGG AACTAAATGG GATTTTGCGA CAGATAAAAT
      F  T  G  W   Y  D  A   K  T  G   G  T  K  W   D  F  A   T  D  K

3241 GCCAGCAGAG GACATCACAT TATACGCGCA GTTTACGATT AATAGTTACA CAGCCACATT
      M  P  A  E   D  I  T   L  Y  A   Q  F  T  I   N  S  Y   T  A  T

3301 TGATATTGAT GGTAAATTAA CGACTCAAAA AGTCACTTAT CAAAGCTTGC TAGAAGAACC
      F  D  I  D   G  K  L   T  T  Q   K  V  T  Y   Q  S  L   L  E  E

3361 AGTAGCGCCA ACGAAGGATG GCTATACGTT CACAGGTTGG TATGATGCTA AAACGGGTGG
      P  V  A  P   T  K  D   G  Y  T   F  T  G  W   Y  D  A   K  T  G

3421 AACTAAATGG GATTTTGCGA CAGGGAAAAT GCCAGCGGGA AATATAACAC TATACGCCCA
      G  T  K  W   D  F  A   T  G  K   M  P  A  G   N  I  T   L  Y  A

3481 GTTCACTAAA AATGACAACC CGAATCCCGA TGATCCAACT ACCAATACCC CAACAGGAAA
      Q  F  T  K   N  D  N   P  N  P   D  D  P  T   T  N  T   P  T  G

3541 TGGTGATGGT ACAAGTAACC CAAGTAATTC AGGAGGCAAT ACCACACTTC CAACAGCTGG
      N  G  D  G   T  S  N   P  S  N   S  G  G  N   T  T  L   P  T  A

3601 TGACGAAAAT ACCATGCTTC CGATTTTTAT CGGGGTTTTC TTGCTAGGAA CAGCGACGCT
      G  D  E  N   T  M  L   P  I  F   I  G  V  F   L  L  G   T  A  T
```

                                                 stop
                                                 inlH

```
3661 AATTCTCCGC AAAACAATCA AAGTAAAATA ACAACAAAAA AAGCTGAGGT CTATAGTTTT
      L  I  L  R   K  T  I   K  V  K   *

3721 TCTATAGGCT TCAGCTTTTT TATTAGTAGA TAAATTCATC TAAACGTCAC AACTAAATCG


3781 TTAACAAGTC TAATTTTAGT GATTAAACGA AATCCTACTA CGCTATAATA TAGTGACTTA
```

                                                  RBS   **start**
                                                                               inlE

```
3841 ACTACGAGAA CATACAAATT TCTAAAAAGC AGAATTACTT TATAAAGGAG ATAGTTTAAT


3901 GAAAAGAAAC AAAACAGCAT TAAGAATCTT AGTCACTTTA GCTGTAGTAT TGGCAATTAC
      M  K  R  N   K  T  A   L  R  I   L  V  T  L   A  V  V   L  A  I

3961 TTTTTGGGTA GGGATGAGCT CAAAAGAAGT ACAAGCAGCG GTGATTGAAC ACCCAACCCC
      T  F  W  V   G  M  S   S  K  E   V  Q  A  A   V  I  E   H  P  T

4021 TATTAACGAA ATTTTTACTG ATCCAGTGCT TACTGATAAT GTAAAAACAC TGCTCGGAAA
      P  I  N  E   I  F  T   D  P  V   L  T  D  N   V  K  T   L  L  G

4081 AGCGGATGTA ACAGACGAAG TTACGCAAAC CGACTTAGAT AGTGTAACTC ATTTATCAGC
      K  A  D  V   T  D  E   V  T  Q   T  D  L  D   S  V  T   H  L  S

4141 AAAATCAGCA GGAATAACAA CAATAGAAGG ATTGCAGTAT CTAACTAATT TATCGGAATT
      A  K  S  A   G  I  T   T  I  E   G  L  Q  Y   L  T  N   L  S  E
```

```
4201 AGAATTAATA GATAATCAAG TAACCGATTT AAATCCTCTT ACTAATTTAA CGAAAATAAC
      L   E   L   I   D   N   Q   V   T   D   L   N   P   L   T   N   L   T   K   I

4261 AGAACTTAGA TTATCTGGAA ATCCGTTAAA AGATGTGAGC GCGCTTGCTG GATTAAAAAA
      T   E   L   R   L   S   G   N   P   L   K   D   V   S   A   L   A   G   L   K

4321 TCTAAAAACG ATGGATCTTA TTTATACAGA TATTACGGAT GTGACACCAC TTGCAGGACT
      N   L   K   T   M   D   L   I   Y   T   D   I   T   D   V   T   P   L   A   G

4381 TTCCAATTTA CAGGTGTTAA ATTTAGATAT CAATCAAATA ACTGATATAA CTCCACTTGC
      L   S   N   L   Q   V   L   N   L   D   I   N   Q   I   T   D   I   T   P   L

4441 AGGACTATCT AATTTACAAT TTTTATCGTT CGGAAGTACT CAAGTAAGTG ATTTGACGCC
      A   G   L   S   N   L   Q   F   L   S   F   G   S   T   Q   V   S   D   L   T

4501 ACTTGCTAAT TTATCTAAAC TAACCACACT AAACGCTATG AATAGTAAAG TAAGCGATGT
      P   L   A   N   L   S   K   L   T   T   L   N   A   M   N   S   K   V   S   D

4561 TTCTCCACTT ACTGGTTTAT CTAATCTCAC AGAAGTTTAT TTGGAAGAGA ATCAAATTAG
      V   S   P   L   T   G   L   S   N   L   T   E   V   Y   L   E   E   N   Q   I

4621 TGATGTGAGT CCGCTTGCAA AATTACCCAA CTTATCTATT GTTACTTTAA CGAATCAAAC
      S   D   V   S   P   L   A   K   L   P   N   L   S   I   V   T   L   T   N   Q

4681 AATCACCAAC CAACCCGTAT TTTATCAAAA TAAACCTATC GTTCCTAATG TAGTAACTGG
      T   I   T   N   Q   P   V   F   Y   Q   N   K   P   I   V   P   N   V   V   T

4741 CCTTTCTGGT GAGCTTATTG CACCGGATAC TATTAGCGAC AATGGAACAT ACACTAGTCC
      G   L   S   G   E   L   I   A   P   D   T   I   S   D   N   G   T   Y   T   S

4801 CAATTTAACG TGGGATTTAA ACAGCTTCAT TAATAGTGTT AGTTACACAT TTAACCAATC
      P   N   L   T   W   D   L   N   S   F   I   N   S   V   S   Y   T   F   N   Q

4861 AGTCACTTTC AAAAATACAA CGGCTCCTTT TAGTGGAACA GTTACACAAC CATTAACAGA
      S   V   T   F   K   N   T   T   A   P   F   S   G   T   V   T   Q   P   L   T

4921 AGTTTACGCT GTAGTTTTTG ATGTGGACGG AGAGCAAACA AGTGCGATGG TAGGTGTGAA
      E   V   Y   A   V   V   F   D   V   D   G   E   Q   T   S   A   M   V   G   V

4981 TGAATTAATT AACGAACCAA CTGCTCCAGC CAAGGAAGGT TATATATTCG ATGGATGGTA
      N   E   L   I   N   E   P   T   A   P   A   K   E   G   Y   I   F   D   G   W

5041 TGACGCAAAA ACAGACGGGA ATAAATGGGA CTTTGGGATA GATAAAATGC CTGCTAGTGA
      Y   D   A   K   T   D   G   N   K   W   D   F   G   I   D   K   M   P   A   S

5101 TATAACTTTA TATGCTAAGT TCACTGAGAA CGAAGAACCA AATGCTAGTA GTCCAATTAA
      D   I   T   L   Y   A   K   F   T   E   N   E   E   P   N   A   S   S   P   I

5161 TGTGGAACCA AATGACAATA ATTCAGACAA TGCAGAACCA AATGCTAGTA GTTCAAATAA
      N   V   E   P   N   D   N   N   S   D   N   A   E   P   N   A   S   S   S   N

5221 TGTACAAGAA AATGGAACTA ACGAAGGAAT AAATAATCTG AACAGTTCAG GTGAAGATAA
      N   V   Q   E   N   G   T   N   E   G   I   N   N   L   N   S   S   G   E   D

5281 AGTCAACATC AAGTTACCGA TTACTGGAGA TAAATTGAAT GTGCTTCCTA TTTTTGTAGG
      K   V   N   I   K   L   P   I   T   G   D   K   L   N   V   L   P   I   F   V
```

                                                                                  stop
                                                                                   inlE

```
5341 AGCAGTTCTT ATCGGAATTG GCTTAGTTTT ATTCCGCAAA AAACGTCAAA CAAAATAAAC
```

```
              G   A   V   L     I   G   I     G   L   V     L   F   R   K     K   R   Q     T   K   *
5401  CAATCAATGG CTTGGCTTCT GTAAATCATG CAGAGACTAA GCCATTTTTT ATTTGGTAAT


5461  TATAAGAAGG AGTTTGCCTT TATAGAGAAC GGGAAAACAT AGAGTGGAAT TCATAGAAAG

                          start
                          dapE
5521  AGGGCGTGAA ATATGGACCA ACAAAAAAAG ATTCAAATTT TAAAGGACTT GGTAAATATT
                        M   D   Q   Q   K   K     I   Q   I     L   K   D     L   V   N   I

5581  GATTCGACTA ATGGGCATGA AGAACAAGTT GCGAACTATT TGCAAAAGTT GTTAGCTGAA
       D   S   T     N   G   H   E     E   Q   V     A   N   Y     L   Q   K     L   L   A   E

5641  CATGGTATTG AGTCCGAAAA GGTACAATAC GACCTAGACA GAGCTAGCCT AGTAAGCGAA
       H   G   I     E   S   E     K   V   Q   Y     D   L   D     R   A   S     L   V   S   E

5701  ATTGGTTCCA GTAACGAGAA GGTTTTGGCA TTTTCAGGGC ATATGGATGT AGTTGATGCG
       I   G   S     S   N   E     K   V   L     A   F   S   G     H   M   D     V   V   D   A

5761  GGTGATGTAT CTAAGTGGAA GTTCCCACCT TTTGAAGCGA CAGAGCATGA AGGGAAACTA
       G   D   V     S   K   W     K   F   P   P     F   E   A     T   E   H     E   G   K   L

5821  TACGGACGCG GCGCAACGGA TATGAAGTCA GGTCTAGCGG CGATGGTTAT TGCAATGATT
       Y   G   R     G   A   T     D   M   K   S     G   L   A     A   M   V     I   A   M   I

5881  GAACTTCATG AAGAAAAACA AAAACTAAAC GGCAAGATCA GATTATTAGC AACAGTTGGG
       E   L   H     E   E   K     Q   K   L   N     G   K   I     R   L   L     A   T   V   G

5941  GAAGAAATCG GTGAACTTGG AGCAGAACAA CTAACACAAA AAGGTTACGC AGATGATTTA
       E   E   I     G   E   L     G   A   E   Q     L   T   Q     K   G   Y     A   D   D   L

6001  GATGGTTTAA TCATCGGCGA ACCGAGTGGA CACAGAATCG TTTATGCGCA TAAAGGTTCC
       D   G   L     I   I   G     E   P   S   G     H   R   I     V   Y   A     H   K   G   S

6061  ATTAATTATA CCGTTAAATC CACTGGTAAA AATGCCCATA GTTCGATGCC GGAATTTGGT
       I   N   Y     T   V   K     S   T   G   K     N   A   H     S   S   M     P   E   F   G

6121  GTGAATGCGA TTGATAACTT GCTGCTATTT TATAATGAAG TAGAAAAATT CGTGAAATCA
       V   N   A     I   D   N     L   L   L   F     Y   N   E     V   E   K     F   V   K   S

6181  ATTGATGCTA CTAACGAAAT ATTAGGCGAT TTTATTCATA ATGTCACCGT AATTGATGGT
       I   D   A     T   N   E     I   L   G   D     F   I   H     N   V   T     V   I   D   G

6241  GGAAATCAAG TCAATAGTAT CCCTGAAAAA GCACAACTGC AAGGGAATAT TCGCTCGATT
       G   N   Q     V   N   S     I   P   E   K     A   Q   L     Q   G   N     I   R   S   I

6301  CCAGAAATGG ATAATGAAAC AGTGAAACAA GTGCTAGTGA AGATTATCAA TAAGTTAAAC
       P   E   M     D   N   E     T   V   K   Q     V   L   V     K   I   I     N   K   L   N

6361  AAACAGGAAA ATGTGAATCT GGAATTAATA TTTGATTATG ATAAACAACC AGTATTTAGT
       K   Q   E     N   V   N     L   E   L   I     F   D   Y     D   K   Q     P   V   F   S

6421  GATAAAAATT CGGATTTAGT CCACATTGCT AAGAGCGTAG CAAGCGACAT TGTCAAAGAA
       D   K   N     S   D   L     V   H   I   A     K   S   V     A   S   D     I   V   K   E

6481  GAAATCCCAT TACTCGGTAT TTCCGGAACA ACCGATGCAG CAGAATTTAC CAAAGCTAAG
       E   I   P     L   L   G     I   S   G   T     T   D   A     A   E   F     T   K   A   K
```

```
6541 AAAGAGTTCC CAGTGATTAT TTTTGGACCA GGAAACGAAA CCCCTCACCA AGTAAACGAA
      K    E   F    P   V   I    I   F   G   P    G   N   E    T   P   H    Q   V   N   E

6601 AATGTTTCTA TAGGAAATTA TTTGGAGATG GTAGATGTTT ACAAACGGAT TGCCACCGAG
      N    V   S    I   G   N    Y   L   E   M    V   D   V    Y   K   R    I   A   T   E

                              stop
                              dapE
6661 TTTTTATCTT GATGAAACTT TAACTTTACT TATTTCCCGA TATAAAATAA GTAATTAAAT
      F    L   S    *

6721 AGAAGTCTAG TATTTGTTTG TAAACAGGTG CTAGGCTTTT TTCTTGCTTT TAATACAGTT


6781 TAGTAC
```

B.2   Nucleotide sequences of the mutant and revertant strains

Nucleotide sequence of *L. monocytogenes* Δ*inlGHE* (S14)

Length of the DNA fragment sequenced: 811 bp. Deletion of most of the *inlGHE* gene cluster. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 AAACGTTACG GCTTCATCTA CGTAGACCAA GATGACTGGG GCAAAGGAAC ATTAGAACGC

 61 TCCCGCAAAG ATTCATTCTT CTGGTATAAA AAAGTAATTG AAACAAATGG TGAAGATTTA

121 GATTAATAGG TAGAGTACAG CCCCGCTTTG GCGGGGCTGT TTTTTTACGC TACTTCCCAC

181 AAGAAACCAA TTCTGCAATC GCTGAACGAA AAAGTTCAAT TAATTGTCAC ATCTTTCGTG

241 TAGAAGGGTC TAATGCGAGA AAAAATGGCG GGCATCTTCT GTTATAATTA TTCATCAAAG

301 ACTAGTAATA TTTTACGCCA AGTCATCCAA GATAGTGCAG ATCCAAGGTG TATTTAATGA

361 TGAATTATCT ACTCAGTCAA ATTATAAGGA GACGATAAAA TGAAACAGAG AAAAACCTCA
                                                     M   K   Q   R   K   T   S

421 GTACTACATG TTTTACTTGT AGTGACAGCT ATCCCGGGTT TATTCCGCAA AAAACGTCAA
       V   L   H   V   L   L   V   V   T   A   I   P   G   L   F   R   K   K   R   Q

481 ACAAAATAAA CCAATCAATG GCTTGGCTTC TGTAAATCAT GCAGAGACTA AGCCATTTTT
       T   K   *

541 TATTTGGTAA TTATAAGAAG GAGTTTGCCT TTATAGAGAA CGGGAAAACA TAGAGTGGAA

601 TTCATAGAAA GAGGGCGTGA AATATGGACC AACAAAAAAA GATTCAAATT TTAAAGGACT

661 TGGTAAATAT TGATTCGACT AATGGGCATG AAGAACAAGT TGCGAACTAT TTGCAAAAGT

721 TGTTAGCTGA ACATGGTATT GAGTCCGAAA AGGTACAATA CGACCTAGAC AGAGCTAGCC

781 TAGTAAGCGA AATTGGTTCC AGTAACGAGA A
```

Nucleotide sequence of *L. monocytogenes* $\Delta inlGHE$ 1-2/8 (S57)

Length of the DNA fragment sequenced: 416 bp. Deletion of most of the *inlGHE* gene cluster. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 TCAATTAATT GTCACATCTT TCGTGTAGAA GGGTCTAATG CGAGAAAAAA TGGCGGGCAT


 61 CTTCTGTTAT AATTATTCAT CAAAGACTAG TAATATTTTA CGCCAAGTCA TCCAAGATAG


121 TGCAGATCCA AGGTGTATTT AATGATGAAT TATCTACTCA GTCAAATTAT AAGGAGACGA


181 TAAAATGAAA CAGAGAAAAA CCTCAGTACT ACATGTTTTA CTTGTAGTGA CAGCTATCCC
           M   K    Q   R   K    T   S   V    L   H   V    L   V   V    T   A   I

241 GGGTTTATTC CGCAAAAAAC GTCAAACAAA ATAAACCAAT CAATGGCTTG GCTTCTGTAA
      P   G   L   F    R   K   K    R   Q   T    K   *

301 ATCATGCAGA GACTAAGCCA TTTTTTATTT GGTAATTATA AGAAGGAGTT TGCCTTTATA


361 GAGAACGGGA AAACATAGAG TGGAATTCAT AGAAAGAGGG CGTGAAATAT GGACCA
```

Nucleotide sequence of *L. monocytogenes* ΔinlGHE 4-3/33 (S58)

Length of the DNA fragment sequenced: 422 bp. Deletion of most of the *inlGHE* gene cluster. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA GAAAAAATGG CGGGCATCTT

 61 CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC CAAGTCATCC AAGATAGTGC

121 AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC AAATTATAAG GAGACGATAA

181 AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT GTAGTGACAG CTATCCCGGG
          M   K   Q    R   K   T    S   V   L    H   V   L   L    V   V   T    A   I   P

241 TTTATTCCGC AAAAAACGTC AAACAAAATA AACCAATCAA TGGCTTGGCT TCTGTAAATC
      G   L   F    R   K   R    Q   T   K    *

301 ATGCAGAGAC TAAGCCATTT TTTATTTGGT AATTATAAGA AGGAGTTTGC CTTTATAGAG

361 AACGGGAAAA CATAGAGTGG AATTCATAGA AAGAGGGCGT GAAATATGGA CCAACAAAAA

421 AA
```

Nucleotide sequence of *L. monocytogenes* ∆*inlGHE* 4-3/55 (S59)

Length of the DNA fragment sequenced: 423 bp. Deletion of most of the *inlGHE* gene cluster. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA GAAAAAATGG CGGGCATCTT

 61 CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC CAAGTCATCC AAGATAGTGC

121 AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC AAATTATAAG GAGACGATAA

181 AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT GTAGTGACAG CTATCCCGGG
        M   K   Q   R   K   T   S   V   L   H   V   L   L   V   V   T   A   I   P

241 TTTATTCCGC AAAAAACGTC AAACAAAATA AACCAATCAA TGGCTTGGCT TCTGTAAATC
     G   L   F   R   K   K   R   Q   T   K   *

301 ATGCAGAGAC TAAGCCATTT TTTATTTGGT AATTATAAGA AGGAGTTTGC CTTTATAGAG

361 AACGGGAAAA CATAGAGTGG AATTCATAGA AAGAGGGCGT GAAATATGGA CCAACAAAAA

421 AAG
```

Nucleotide sequence of *L. monocytogenes* ∆*inlG*I, FG2 (S38)

Length of the DNA fragment sequenced: 631 bp. Deletion of the *inlG* gene not in frame.
Resulting mutant gene codes for a 22-amino acid residual peptide.

```
  1 AGCCCCGCTT TGGCGGGGCT GTTTTTTTAC GCTACTTCCC ACAAGAAACC AATTCTGCAA


 61 TCGCTGAACG AAAAAGTTCA ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA


121 GAAAAAATGG CGGGCATCTT CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC


181 CAAGTCATCC AAGATAGTGC AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC


241 AAATTATAAG GAGACGATAA AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT
                                    M   K   Q   R   K   T   S   V   L   H   V   L   L

301 GTAGTGACAG CTATCCGGGT GCAATACTAA TCTTACGAAA AAAAACTACT AACATTTAAG
     V   V   T   A   I   R   V   Q   Y   *

361 ATAAAGTAGA TTTGGTTTCT ATTTTCAGTA GAGACCAAAT TTTTTTGTTA ATTTGGTCTA


421 AAAAAGGGTA TCTATTATTA ATGACTTATT TAGAAGAATA ATTAGTGAAT CTAATTTAAA


481 TAAAAGGAGA GGGATTATGA AAAAACGGTG GAATTCAGTA TTCAAACTAG TTTTAATGGT


541 AACTGCTATT CTCGGGCTTA GCCTATATGT AACGACAAGC CAAGGTGTGG AGGTTCGGGC


601 AGAGAGCATC ACGCAGCCAA CCGCAATTAA T
```

Nucleotide sequence of *L. monocytogenes* ∆*inlG*I, FG12 (S37)

Length of the DNA fragment sequenced: 631 bp. Deletion of the *inlG* gene not in frame. Resulting mutant gene codes for a 22-amino acid residual peptide.

```
  1 AGCCCCGCTT TGGCGGGGCT GTTTTTTTAC GCTACTTCCC ACAAGAAACC AATTCTGCAA

 61 TCGCTGAACG AAAAAGTTCA ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA

121 GAAAAAATGG CGGGCATCTT CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC

181 CAAGTCATCC AAGATAGTGC AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC

241 AAATTATAAG GAGACGATAA AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT
                                    M  K  Q   R  K  T   S  V  L   H  V  L  L

301 GTAGTGACAG CTATCCGGGT GCAATACTAA TCTTACGAAA AAAAACTACT AACATTTAAG
     V  V  T   A  I  R   V  Q  Y  *

361 ATAAAGTAGA TTTGGTTTCT ATTTTCAGTA GAGACCAAAT CTTTTTGTTA ATTTGGTCTA

421 AAAAAGGGTA TCTACTATTA ATGACTTATT TAGAAGAATA ATCAGTGAAT CTAATTTAAA

481 TAAAAGGAGA GGGATTATGA AAAAACGGTG GAATTCAGTA TTCAAACTAG TTTTAATGGT

541 AACTGCTATT CTCGGGCTTA GCCTATATGT AACGACAAGC CAAGGTGTGG AGGTTCGGGC

601 AGAGAGCATC ACGCAGCCAA CCGCAATTAA T
```

Nucleotide sequence of *L. monocytogenes* ∆*inlG*II, G4-3 (S53)

Length of the DNA fragment sequenced: 627 bp. In-frame deletion of most *inlG* gene.
Resulting mutant gene codes for a 32-amino acid residual peptide.

```
  1 TCTAGATTAA TAGGTAGAGT ACAGCCCCGC TTTGGCGGGG CTGTTTTTTT ACGCTACTTC


 61 CCACAAGAAA CCAATTCTGC AATCGCTGAA CGAAAAAGTT CAATTAATTG TCACATCTTT


121 CGTGTAGAAG GGTCTAATGC GAGAAAAAAT GGCGGGCATC TTCTGTTATA ATTATTCATC


181 AAAGACTAGT AATATTTTAC GCCAAGTCAT CCAAGATAGT GCAGATCCAA GGTGTATTTA


241 ATGATGAATT ATCTACTCAG TCAAATTATA AGGAGACGAT AAAATGAAAC AGAGAAAAAC
                                                    M   K    Q   R   K

301 CTCAGTACTA CATGTTTTAC TTGTAGTGAC AGCTATCCCG GGTGCAATAC TAATCTTACG
      T   S   V   L    H   V   L    L   V   V    T   A   I   P    G   A   I    L   I   L

361 AAAAAAAACT ACTAACATTT AAGATAAAGT AGATTTGGTT TCTATTTTCA GTAGAGACCA
      R   K   K   T    T   N   I    *

421 AATTTTTTTG TTAATTTGGT CTAAAAAAGG GTATCTATTA TTAATGACTT ATTTAGAAGA


481 ATAATTAGTG AATCTAATTT AAATAAAAGG AGAGGGATTA TGAAAAAACG GTGGAATTCA


541 GTATTCAAAC TAGTTTTAAT GGGTAACTGC TATTCTCGGG CTTAGCCTAT ATGTAACGAC


601 AAGCCAAGGT GTGGAGGTTC GGGCAGA
```

Nucleotide sequence of *L. monocytogenes* Δ*inlG*II, G5-3 (S54)

Length of the DNA fragment sequenced: 606 bp. In-frame deletion of most *inlG* gene. Resulting mutant gene codes for a 32-amino acid residual peptide.

```
  1 AGCCCCGCTT TGGCGGGGCT GTTTTTTTAC GCTACTTCCC ACAAGAAACC AATTCTGCAA

 61 TCGCTGAACG AAAAAGTTCA ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA

121 GAAAAAATGG CGGGCATCTT CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC

181 CAAGTCATCC AAGATAGTGC AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC

241 AAATTATAAG GAGACGATAA AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT
                                   M   K   Q   R   K   T   S   V   L   H   V   L   L

301 GTAGTGACAG CTATCCCGGG TGCAATACTA ATCTTACGAA AAAAAACTAC TAACATTTAA
      V   V   T   A   I   P   G   A   I   L   I   L   R   K   K   T   T   N   I   *

361 GATAAAGTAG ATTTGGTTTC TATTTTCAGT AGAGACCAAA TTTTTTTGTT AATTTGGTCT

421 AAAAAAGGGT ATCTATTATT AATGACTTAT TTAGAAGAAT AATTAGTGAA TCTAATTTAA

481 ATAAAAGGAG AGGGATTATG AAAAAACGGT GGAATTCAGT ATTCAAACTA GTTTTAATGG

541 TAACTGCTAT TCTCGGGCTT AGCCTATATG TAACGACAAG CCAAGGTGTG GAGGTTCGGG

601 CAGAGA
```

Nucleotide sequence of *L. monocytogenes* Δ*inlH*, H3-2 (S41)

Length of the DNA fragment sequenced: 671 bp. In-frame deletion of most *inlH* gene.
Resulting mutant gene codes for a 47-amino acid residual peptide.

```
  1 AAGTATGATT CCTACTATTT TAGGAACGCT TTTCATCGGA GGTGCAATAC TAATCTTACG


 61 AAAAAAAACT ACTAACATTT AAGATAAAGT AGATTTGGTT TCTATTTTCA GTAGAGACCA


121 AATTTTTTTG TTAATTTGGT CTAAAAAAGG GTATCTATTA TTAATGACTT ATTTAGAAGA


181 ATAATTAGTG AATCTAATTT AAATAAAAGG AGAGGGATTA TGAAAAAACG GTGGAATTCA
                                                 M  K  K    R  W  N  S

241 GTATTCAAAC TAGTTTTAAT GGTAACTGCT ATTCTCGGGC TTAGCCTATA TGTAACGACA
     V  F  K   L  V  L    M  V  T  A   I  L  G    L  S  L    Y  V  T  T

301 AGCCCGGGTT TCTTGCTAGG AACAGCGACG CTAATTCTCC GCAAAACAAT CAAAGTAAAA
     S  P  G   F  L  L    G  T  A  T   L  I  L    R  K  T    I  K  V  K

361 TAACAACAAA AAAAGCTGAG GTCTATAGTT TTTCTATAGG CTTCAGCTTT TTTATTAGTA
     *

421 GATAAATTCA TCTAAACGTC ACAACTAAAT CGTTAACAAG TCTAATTTTA GTGATTAAAC


481 GAAATCCTAC TACGCTATAA TATAGTGACT TAACTACGAG AACATACAAA TTTCTAAAAA


541 GCAGAATTAC TTTATAAAGG AGATAGTTTA ATGAAAAGAA ACAAAACAGC ATTAAGAATC


601 TTAGTCACTT TAGCTGTAGT ATTGGCAATT ACTTTTTGGG TAGGGATGAG CTCAAAAGAA


661 GTACAAGCAG C
```

Nucleotide sequence of *L. monocytogenes* ΔinlH, H3-4 (S42)

Length of the DNA fragment sequenced: 671 bp. In-frame deletion of most *inlH* gene. Resulting mutant gene codes for a 47-amino acid residual peptide.

```
  1 AAGTATGATT CCTACTATTT TAGGAACGCT TTTCATCGGA GGTGCAATAC TAATCTTACG


 61 AAAAAAAACT ACTAACATTT AAGATAAAGT AGATTTGGTT TCTATTTTCA GTAGAGACCA


121 AATTTTTTTG TTAATTTGGT CTAAAAAAGG GTATCTATTA TTAATGACTT ATTTAGAAGA


181 ATAATTAGTG AATCTAATTT AAATAAAAGG AGAGGGATTA TGAAAAAACG GTGGAATTCA
                                                 M  K  K   R  W  N  S

241 GTATTCAAAC TAGTTTTAAT GGTAACTGCT ATTCTCGGGC TTAGCCTATA TGTAACGACA
        V  F  K   L  V  L   M  V  T  A   I  L  G   L  S  L   Y  V  T  T

301 AGCCCGGGTT TCTTGCTAGG AACAGCGACG CTAATTCTCC GCAAAACAAT CAAAGTAAAA
        S  P  G   F  L  L   G  T  A  T   L  I  L   R  K  T   I  K  V  K

361 TAACAACAAA AAAAGCTGAG GTCTATAGTT TTTCTATAGG CTTCAGCTTT TTTATTAGTA
        *

421 GATAAATTCA TCTAAACGTC ACAACTAAAT CGTTAACAAG TCTAATTTTA GTGATTAAAC


481 GAAATCCTAC TACGCTATAA TATAGTGACT TAACTACGAG AACATACAAA TTTCTAAAAA


541 GCAGAATTAC TTTATAAAGG AGATAGTTTA ATGAAAAGAA ACAAAACAGC ATTAAGAATC


601 TTAGTCACTT TAGCTGTAGT ATTGGCAATT ACTTTTTGGG TAGGGATGAG CTCAAAAGAA


661 GTACAAGCAG C
```

Nucleotide sequence of *L. monocytogenes* Δ*inlE*, E2-1 (S39)

Length of the DNA fragment sequenced: 784 bp. In-frame deletion of most *inlE* gene.
Resulting mutant gene codes for a 49-amino acid residual peptide.

```
  1 CTTCCAACAG CTGGTGACGA AAATACCATG CTTCCGATTT TTATCGGGGT TTTCTTGCTA


 61 GGAACAGCGA CGCTAATTCT CCGCAAAACA ATCAAAGTAA AATAACAACA AAAAAAGCTG


121 AGGTCTATAG TTTTTCTATA GGCTTCAGCT TTTTTATTAG TAGATAAATT CATCTAAACG


181 TCACAACTAA ATCGTTAACA AGTCTAATTT TAGTGATTAA ACGAAATCCT ACCACGCTAT


241 AATATAGTGA CTTAACTACG AGAACATACA AATTTCTAAA AAGCAGAATT ACTTTATAAA


301 GGAGATAGTT TAATGAAAAG AAACAAAACA GCATTAAGAA TCTTAGTCAC TTCAGCTGTA
                    M   K   R   N   K   T   A   L   R   I   L   V   T   S   A   V

361 GTATTGGCAA TTACTTTTTG GGTAGGGATG AGCTCAAAAG AAGTACAAGC AGCGGTGATT
         V   L   A   I   T   F   W   V   G   M   S   S   K   E   V   Q   A   A   V   I

421 GAACACCCGG GTTTATTCCG CAAAAAACGT CAAACAAAT AAACCAATCA ATGGCTTGGC
      E   H   P   G   L   F   R   K   K   R   Q   T   K   *

481 TTCTGTAAAT CATGCAGAGA CTAAGCCATT TTTTATTTGG TAATTATAAG AAGGAGTTTG


541 CCTTTATAGA GAACGGGAAA ACATGGAGTG GAATTCATAG AAAGAGGGCG TGAAATATGG


601 ACCAACAAAA AAAGATTCAA ATTTTAAAGG ACTTGGTAGA TATTGATTCG ACTAATGGGC


661 ATGAAGAACA AGTTGCGAAC TATTTGCAAA AGTTGTTAGC TGAACATGGT ATTGAGTCCG


721 AAAAGGTACA ATACGACCTA GACAGAGCTA GCCTAGTAAG CGAAATTGGT TCCAGTAACG


781 AGAA
```

Nucleotide sequence of *L. monocytogenes* ∆*inlE*, E6-4 (S40)

Length of the DNA fragment sequenced: 779 bp. In-frame deletion of most *inlE* gene. Resulting mutant gene codes for a 49-amino acid residual peptide.

```
  1 AACAGCTGGT GACGAAAATA CCATGCTTCC GATTTTTATC GGGGTTTTCT TGCTAGGAAC

 61 AGCGACGCTA ATTCTCCGCA AAACAATCAA AGTAAAATAA CAACAAAAAA AGCTGAGGTC

121 TATAGTTTTT CTATAGGCTT CAGCTTTTTT ATTAGTAGAT AAATTCATCT AAACGTCACA

181 ACTAAATCGT TAACAAGTCT AATTTTAGTG ATTAAACGAA ATCCTACTAC GCTATAATAT

241 AGTGACTTAA CTACGAGAAC ATACAAATTT CTAAAAAGCA GAATTACTTT ATAAAGGAGA

301 TAGTTTAATG AAAAGAAACA AAACAGCATT AAGAATCTTA GTCACTTTAG CTGTAGTATT
              M    K    R    N    K    T    A    L    R    I    L    V    T    L    A    V    V

361 GGCAATTACT TTTTGGGTAG GGATGAGCTC AAAAGAAGTA CAAGCAGCGG TGATTGAACA
    L    A    I    T    F    W    V    G    M    S    S    K    E    V    Q    A    A    V    I    E

421 CCCGGGTTTA TTCCGCAAAA AACGTCAAAC AAAATAAACC AATCAATGGC TTGGCTTCTG
    H    P    G    L    F    R    K    K    R    Q    T    K    *

481 TAAATCATGC AGAGACTAAG CCATTTTTTA TTTGGTAATT ATAAGAAGGA GTTTGCCTTT

541 ATAGAGAACG GGAAACATG  GAGTGGAATT CATAGAAAGA GGGCGTGAAA TATGGACCAA

601 CAAAAAAGA  TTCAAATTTT AAAGGACTTG GTAGATATTG ATTCGACTAA TGGGCATGAA

661 GAACAAGTTG CGAACTATTT GCAAAAGTTG TTAGCTGAAC ATGGTATTGA GTCCGAAAAG

721 GTACAATACG ACCTAGACAG AGCTAGCCTA GTAAGCGAAA TTGGTTCCAG TAACGAGAA
```

Nucleotide sequence of *L. monocytogenes inlG*II$^+$, C2-2/34 (S62)

Length of the DNA fragment sequenced: 758 bp. Insertion of wild type *inlG* confirmed.

```
  1 TTCAATTAAT TGTCACATCT TTCGTGTAGA AGGGTCTAAT GCGAGAAAAA ATGGCGGGCA


 61 TCTTCTGTTA TAATTATTCA TCAAAGACTA GTAATATTTT ACGCCAAGTC ATCCAAGATA


121 GTGCAGATCC AAGGTGTATT TAATGATGAA TTATCTACTC AGTCAAATTA TAAGGAGACG


181 ATAAAATGAA ACAGAGAAAA ACCTCAGTAC TACATGTTTT ACTTGTAGTG ACAGCTATCT
             M   K   Q   R   K   T   S   V   L   H   V   L   L   V   V   T   A   I

241 TGGGAATTAG TTTATGGGTA AATGCAAGTC ATGGGATGAA AGCTCAGGCA GAGAGTATTG
     L   G   I   S   L   W   V   N   A   S   H   G   M   K   A   Q   A   E   S   I

301 CGCAACCAGC GCCAATTAAC GAAATTTTCA CGGATCCAGC ATTAGCGGAC GAGGTGAAGA
     A   Q   P   A   P   I   N   E   I   F   T   D   P   A   L   A   D   E   V   K

361 CGGNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
     T

421 NAATTAAAGC TAACGACAGC GCAGATGCGA CGAGTACTAA ACTTCCAAAA ACAAGTGATG
         I   K   A   N   D   S   A   D   A   T   S   T   K   L   P   K   T   S   D

481 ATTCAAGTAT GATTCCTACT ATTTTAGGAA CGCTTTTCAT CGGAGGTGCA ATACTAATCT
     D   S   S   M   I   P   T   I   L   G   T   L   F   I   G   G   A   I   L   I

541 TACGAAAAAA AACTACTAAC ATTTAAGATA AAGTAGATTT GGTTTCTATT TTCAGTAGAG
     L   R   K   K   T   T   N   I   *

601 ACCAAATTTT TTTGTTAATT TGGTCTAAAA AAGGGTATCT ATTATTAATG ACTTATTTAG


661 AAGAATAATT AGTGAATCTA ATTTAAATAA AAGGAGAGGG ATTATGAAAA AACGGTGGAA


721 TTCAGTATTC AAACTAGTTT TAATGGTAAC TGCTATTCTC GGGCTTAGCC TATATGTAAC


781 GACAAGCCAA GGTGTGGAGG TTCGGGCAGA GAGCAT
```

Nucleotide sequence of *L. monocytogenes inlH*$^+$, H17 (S79)

Length of the DNA fragment sequenced: 823 bp. Insertion of wild type *inlH* confirmed.

```
  1 AACAAGTGAT GATTCAAGTA TGATTCCTAC TATTTTAGGA ACGCTTTTCA TCGGAGGTGC

 61 AATACTAATC TTACGAAAAA AAACTACTAA CATTTAAGAT AAAGTAGATT TGGTTTCTAT

121 TTTCAGTAGA GACCAAATTT TTTTGTTAAT TTGGTCTAAA AAAGGGTATC TATTATTAAT

181 GACTTATTTA GAAGAATAAT TAGTGAATCT AATTTAAATA AAAGGAGAGG GATTATGAAA
                                                               M   K

241 AAACGGTGGA ATTCAGTATT CAAACTAGTT TTAATGGTAA CTGCTATTCT CGGGCTTAGC
     K   R   W   N   S   V   F   K   L   V   L   M   V   T   A   I   L   G   L   S

301 CTATATGTAA CGACAAGCCA AGGTGTGGAG GTTCGGGCAG AGAGCATCAC GCAGCCAACC
     L   Y   V   T   T   S   Q   G   V   E   V   R   A   E   S   I   T   Q   P   T

361 GCAATTAATG TGATTTTCCC TGATCCAGCT CTTGCGAATG CANNNNNNNN NNNNNNNNNN
     A   I   N   V   I   F   P   D   P   A   L   A   N   A

421 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN

481 AATACCCCAA CAGGAAATGG TGATGGTACA AGTAACCCAA GTAATTCAGG AGGCAATACC
     N   T   P   T   G   N   G   D   G   T   S   N   P   S   N   S   G   G   N   T

541 ACACTTCCAA CAGCTGGTGA CGAAAATACC ATGCTTCCGA TTTTTATCGG GGTTTTCTTG
     T   L   P   T   A   G   D   E   N   T   M   L   P   I   F   I   G   V   F   L

601 CTAGGAACAG CGACGCTAAT TCTCCGCAAA ACAATCAAAG TAAAATAACA ACAAAAAAAG
     L   G   T   A   T   L   I   L   R   K   T   I   K   V   K   *

661 CTGAGGTCTA TAGTTTTTCT ATAGGCTTCA GCTTTTTTAT TAGTAGATAA ATTCATCTAA

721 ACGTCACAAC TAAATCGTTA ACAAGTCTAA TTTTAGTGAT TAAACGAAAT CCTACTACGC

781 TATAATATAG TGACTTAACT ACGAGAACAT ACAAATTTCT AAAAAGCAGA ATTACTTTAT

841 AAAGGAGATA GTTTAATGAA AAGAAACAAA ACAGCATTAA GAATCTTAGT CACTTTAGCT

901 G
```

Nucleotide sequence of *L. monocytogenes inlH$^+$*, H19 (S80)

Length of the DNA fragment sequenced: 763 bp. Insertion of wild type *inlH* confirmed.

```
  1 AACAAGTGAT GATTCAAGTA TGATTCCTAC TATTTTAGGA ACGCTTTTCA TCGGAGGTGC


 61 AATACTAATC TTACGAAAAA AAACTACTAA CATTTAAGAT AAAGTAGATT TGGTTTCTAT


121 TTTCAGTAGA GACCAAATTT TTTTGTTAAT TTGGTCTAAA AAAGGGTATC TATTATTAAT


181 GACTTATTTA GAAGAATAAT TAGTGAATCT AATTTAAATA AAAGGAGAGG GATTATGAAA
                                                                 M   K

241 AAACGGTGGA ATTCAGTATT CAAACTAGTT TTAATGGTAA CTGCTATTCT CGGGCTTAGC
     K   R   W   N   S   V   F   K   L   V   L   M   V   T   A   I   L   G   L   S

301 CTATATGTAA CGACAAGCCA AGGTGTGGAG GTTCGGGCAG AGAGCATCAC GCAGCCAACC
     L   Y   V   T   T   S   Q   G   V   E   V   R   A   E   S   I   T   Q   P   T

361 GCAATTAATG TGATTTTCCC TGATCCAGCT CTTGCGAATG CANNNNNNNN NNNNNNNNNN
     A   I   N   V   I   F   P   D   P   A   L   A   N   A

421 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN


481 ACACTTCCAA CAGCTGGTGA CGAAAATACC ATGCTTCCGA TTTTTATCGG GGTTTTCTTG
     T   L   P   T   A   G   D   E   N   T   M   L   P   I   F   I   G   V   F   L

541 CTAGGAACAG CGACGCTAAT TCTCCGCAAA ACAATCAAAG TAAAATAACA ACAAAAAAAG
     L   G   T   A   T   L   I   L   R   K   T   I   K   V   K   *

601 CTGAGGTCTA TAGTTTTTCT ATAGGCTTCA GCTTTTTTAT TAGTAGATAA ATTCATCTAA


661 ACGTCACAAC TAAATCGTTA ACAAGTCTAA TTTTAGTGAT TAAACGAAAT CCTACTACGC


721 TATAATATAG TGACTTAACT ACGAGAACAT ACAAATTTCT AAAAAGCAGA ATTACTTTAT


781 AAAGGAGATA GTTTAATGAA AAGAAACAAA ACAGCATTAA GAATCTTAGT CACTTTAGCT


841 G
```

Nucleotide sequence of *L. monocytogenes inlE*⁺, E4-2/4 (S70)

Length of the DNA fragment sequenced: 1,172 bp. Insertion of wild type *inlE* confirmed.

```
   1 CGGGGTTTTC TTGCTAGGAA CAGCGACGCT AATTCTCCGC AAAACAATCA AAGTAAAATA


  61 ACAACAAAAA AAGCTGAGGT CTATAGTTTT TCTATAGGCT TCAGCTTTTT TATTAGTAGA


 121 TAAATTCATC TAAACGTCAC AACTAAATCG TTAACAAGTC TAATTTTAGT GATTAAACGA


 181 AATCCTACTA CGCTATAATA TAGTGACTTA ACTACGAGAA CATACAAATT TCTAAAAAGC


 241 AGAATTACTT TATAAAGGAG ATAGTTTAAT GAAAAGAAAC AAAACAGCAT TAAGAATCTT
                                        M  K  R  N   K  T  A  L   R  I

 301 AGTCACTTTA GCTGTAGTAT TGGCAATTAC TTTTTGGGTA GGGATGAGCT CAAAAGAAGT
      L  V  T  L   A  V  V   L  A  I   T  F  W  V   G  M  S   S  K  E

 361 ACAAGCAGCG GTGATTGAAC ACCCAACCCC TATTAACGAA ATTTTTACTG ATCCAGTGCT
      V  Q  A  A   V  I  E   H  P  T   P  I  N  E   I  F  T   D  P  V

 421 TACTGATAAT GTAAAAACAC TGCTCGGAAA AGCGGATGTA ACAGACGAAG TTACGCAAAC
      L  T  D  N   V  K  T   L  L  G   K  A  D  V   T  D  E   V  T  Q

 481 CGACTTAGAT AGTGTAACTC ATTTATCAGC AAAATCAGCA GGAATAACAA CAATAGAAGG
      T  D  L  D   S  V  T   H  L  S   A  K  S  A   G  I  T   T  I  E

 541 ATTGCAGTAT CTAACTAATT TATCGGAATT AGAATTAATA GATAATCAAG TAACCGATTT
      G  L  Q  Y   L  T  N   L  S  E   L  E  L  I   D  N  Q   V  T  D

 601 AAATCCTCTT ACTAATTTAA CGNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
      L  N  P  L   T  N  L   T

 661 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN


 721 TAATTCAGAC AATGCAGAAC CAAATGCTAG TAGTTCAAAT AATGTACAAG AAAATGGAAC
       N  S  D   N  A  E   P  N  A   S  S  S  N   N  V  Q   E  N  G

 781 TAACGAAGGA ATAAATAATC TGAACAGTTC AGGTGAAGAT AAAGTCAACA TCAAGTTACC
      T  N  E  G   I  N  N   L  N  S   S  G  E  D   K  V  N   I  K  L

 841 GATTACTGGA GATAAATTGA ATGTGCTTCC TATTTTTGTA GGAGCAGTTC TTATCGGAAT
      P  I  T  G   D  K  L   N  V  L   P  I  F  V   G  A  V   L  I  G

 901 TGGCTTAGTT TTATTCCGCA AAAAACGTCA AACAAAATAA ACCAATCAAT GGCTTGGCTT
      I  G  L  V   L  F  R   K  K  R   Q  T  K  *

 961 CTGTAAATCA TGCAGAGACT AAGCCATTTT TTATTTGGTA ATTATAAGAA GGAGTTTGCC


1021 TTTATAGAGA ACGGGAAAAC ATAGAGTGGA ATTCATAGAA AGAGGGCGTG AAATATGGAC


1081 CAACAAAAAA AGATTCAAAT TTTAAAGGAC TTGGTAAATA TTGATTCGAC TAATGGGCAT
```

```
1141 GAAGAACAAG TTGCGAACTA TTTGCAAAAG TTGTTAGCTG AACATGGTAT TGAGTCCGAA

1201 AAGGTACAAT ACGACCTAGA CAGAGCTAGC CTAGTAAGCG AAATTGGTTC CAGTAACGAG

1261 AAGGTTTTGG CATTTTCAGG
```

Nucleotide sequence of *L. monocytogenes* Δ*inlA/GHE*, A3-11 (S28)

Length of the DNA fragment sequenced: 673 bp. Deletion of most of the *inlGHE* gene cluster. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 AGCCCCGCTT TGGCGGGGCT GTTTTTTTAC GCTACTTCCC ACAAGAAACC AATTCTGCAA

 61 TCGCTGAACG AAAAAGTTCA ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA

121 GAAAAAATGG CGGGCATCTT CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC

181 CAAGTCATCC AAGATAGTGC AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC

241 AAATTATAGG GAGACGATAA AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT
                                  M   K   Q   R   K   T   S   V   L   H   V   L   L
301 GTAGTGACAG CTATCCCGGG TTTATTCCGC AAAAAACGTC AAACAAAATA AACCAATCAA
      V   V   T   A   I   P   G   L   F   R   K   K   R   Q   T   K   *
361 TGGCTTGGCT TCTGTAAATC ATGCAGAGAC TAAGCCATTT TTTATTTGGT AATTATAAGA

421 AGGAGTTTGC CTTTATAGAG AACGGGAAAA CATAGAGTGG AATTCATAGA AAGAGGGCGT

481 GAAATATGGA CCAACAAAAA AAGATTCAAA TTTTAAAGGA CTTGGTAAAT ATTGATTCGA

541 CTAATGGGCA TGAAGAACAA GTTGCGAACT ATTTGCAAAA GTTGTTAGCT GAACATGGTA

601 TTGAGTCCGA AAAGGTACAA TACGACCTAG ACAGAGCTAG CCTAGTAAGC GAAATTGGTT

661 CCAGTAACGA GAA
```

Nucleotide sequence of *L. monocytogenes* Δ*inlB/GHE*, B4-4/6 (S67)

Length of the DNA fragment sequenced: 673 bp. Deletion of most of the *inlGHE* gene cluster. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 AGCCCCGCTT TGGCGGGGCT GTTTTTTTAC GCTACTTCCC ACAAGAAACC AATTCTGCAA

 61 TCGCTGAACG AAAAAGTTCA ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA

121 GAAAAAATGG CGGGCATCTT CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC

181 CAAGTCATCC AAGATAGTGC AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC

241 AAATTATAAG GAGACGATAA AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT
                                   M   K   Q   R   K   T   S   V   L   H   V   L   L

301 GTAGTGACAG CTATCCCGGG TTTATTCCGC AAAAAACGTC AAACAAAATA AACCAATCAA
       V   V   T   A   I   P   G   L   F   R   K   K   R   Q   T   K   *

361 TGGCTTGGCT TCTGTAAATC ATGCAGAGAC TAAGCCATTT TTTATTTGGT AATTATAAGA

421 AGGAGTTTGC CTTTATAGAG AACGGGAAAA CATAGAGTGG AATTCATAGA AAGAGGGCGT

481 GAAATATGGA CCAACAAAAA AAGATTCAAA TTTTAAAGGA CTTGGTAAAT ATTGATTCGA

541 CTAATGGGCA TGAAGAACAA GTTGCGAACT ATTTGCAAAA GTTGTTAGCT GAACATGGTA

601 TTGAGTCCGA AAAGGTACAA TACGACCTAG ACAGAGCTAG CCTAGTAAGC GAAATTGGTT

661 CCAGTAACGA GAA
```

Nucleotide sequence of *L. monocytogenes* Δ*inlB/GHE*, B4-6/11 (S68)

Length of the DNA fragment sequenced: 702 bp. Deletion of most of the *inlGHE* gene cluster. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 GATTAATAGG TAGAGTACAG CCCCGCTTTG GCGGGGCTGT TTTTTTACGC TACTTCCCAC

 61 AAGAAACCAA TTCTGCAATC GCTGAACGAA AAAGTTCAAT TAATTGTCAC ATCTTTCGTG

121 TAGAAGGGTC TAATGCGAGA AAAAATGGCG GGCATCTTCT GTTATAATTA TTCATCAAAG

181 ACTAGTAATA TTTTACGCCA AGTCATCCAA GATAGTGCAG ATCCAAGGTG TATTTAATGA

241 TGAATTATCT ACTCAGTCAA ATTATAAGGA GACGATAAAA TGAAACAGAG AAAAACCTCA
                                                     M   K   Q   R   K   T   S

301 GTACTACATG TTTTACTTGT AGTGACAGCT ATCCCGGGTT TATTCCGCAA AAAACGTCAA
      V   L   H   V   L   L   V   V   T   A   I   P   G   L   F   R   K   K   R   Q

361 ACAAAATAAA CCAATCAATG GCTTGGCTTC TGTAAATCAT GCAGAGACTA AGCCATTTTT
      T   K   *

421 TATTTGGTAA TTATAAGAAG GAGTTTGCCT TTATAGAGAA CGGGAAAACA TAGAGTGGAA

481 TTCATAGAAA GAGGGCGTGA AATATGGACC AACAAAAAAA GATTCAAATT TTAAAGGACT

541 TGGTAAATAT TGATTCGACT AATGGGCATG AAGAACAAGT TGCGAACTAT TTGCAAAAGT

601 TGTTAGCTGA ACATGGTATT GAGTCCGAAA AGGTACAATA CGACCTAGAC AGAGCTAGCC

661 TAGTAAGCGA AATTGGTTCC AGTAACGAGA AGGTTTTGGC AT
```

Nucleotide sequence of *L. monocytogenes* ∆*inlC/GHE*, C11-3 (S29)

Length of the DNA fragment sequenced: 673 bp. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 AGCCCCGCTT TGGCGGGGCT GTTTTTTTAC GCTACTTCCC ACAAGAAACC AATTCTGCAA

 61 TCGCTGAACG AAAAAGTTCA ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA

121 GAAAAAATGG CGGGCATCTT CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC

181 CAAGTCATCC AAGATAGTGC AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC

241 AAATTATAAG GAGACGATAA AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT
                                   M   K   Q   R   K   T   S   V   L   H   V   L   L

301 GTAGTGACAG CTGTCCCGGG TTTATTCCGC AAAAAACGTC AAACAAAATA AACCAATCAA
     V   V   T   A   V   P   G   L   F   R   K   K   R   Q   T   K   *

361 TGGCTTGGCT TCTGTAAATC ATGCAGAGAC TAAGCCATTT TTCATTTGGT AATTATAAGA

421 AGGAGTTTGC CTTTATAGAG AACGGGAAAA CATAGAGTGG AATTCATAGA AAGAGGGCGT

481 GAAATATGGA CCAACAAAAA AAGATTCAAA TTTTAAAGGA CTTGGTAAAT ATTGATTCGA

541 CTAATGGGCA TGAAGAACAA GTTGCGAACT ATTTGCAAAA GTTGTTAGCT GAACATGGTA

601 TTGAGTCCGA AAAGGTACAA TACGACCTAG ACAGAGCTAG CCTAGTAAGC GAAATTGGTT

661 CCAGTAACGA GAA
```

Nucleotide sequence of *L. monocytogenes* Δ*inlA/B/GHE*, AB12 (S34)

Length of the DNA fragment sequenced: 673 bp. Deletion of most of the *inlGHE* gene cluster. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 AGCCCCGCTT TGGCGGGGCT GTTTTTTTAC GCTACTTCCC ACAAGAAACC AATTCTGCAA

 61 TCGCTGAACG AAAAAGTTCA ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA

121 GAAAAAATGG CGGGCATCTT CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC

181 CAAGTCATCC AAGATAGTGC AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC

241 AAATTATAAG GAGACGATAA AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT
                                  M   K   Q   R   K   T   S   V   L   H   V   L   L

301 GTAGTGACAG CTATCCCGGG TTTATTCCGC AAAAAACGTC AAACAAAATA AACCAATCAA
      V   V   T   A   I   P   G   L   F   R   K   K   R   Q   T   K   *

361 TGGCTTGGCT TCTGTAAATC ATGCAGAGAC TAAGCCATTT TTTATTTGGT AATTATAAGA

421 AGGAGTTTGC CTTTATAGAG AACGGGAAAA CATAGAGTGG AATTCATAGA AAGAGGGCGT

481 GAAATATGGA CCAACAAAAA AAGATTCAAA TTTTAAAGGA CTTGGTAAAT ATTGATTCGA

541 CTAATGGGCA TGAAGAACAA GTTGCGAACT ATTTGCAAAA GTTGTTAGCT GAACATGGTA

601 TTGAGTCCGA AAAGGTACAA TACGACCTAG ACAGAGCTAG CCTAGTAAGC GAAATTGGTT

661 CCAGTAACGA GAA
```

Nucleotide sequence of *L. monocytogenes* Δ*inlA/B/GHE*, AB14 (S35)

Length of the DNA fragment sequenced: 673 bp. Deletion of most of the *inlGHE* gene cluster. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 AGCCCCGCTT TGGCGGGGCT GTTTTTTTAC GCTACTTCCC ACAAGAAACC AATTCTGCAA

 61 TCGCTGAACG AAAAAGTTCA ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA

121 GAAAAAATGG CGGGCATCTT CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC

181 CAAGTCATCC AAGATAGTGC AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC

241 AAATTATAAG GAGACGATAA AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT
                                   M   K   Q   R   K   T   S   V   L   H   V   L   L

301 GTAGTGACAG CTATCCCGGG TTTATTCCGC AAAAAACGTC AAACAAAATA AACCAATCAA
       V   V   T   A   I   P   G   L   F   R   K   K   R   Q   T   K   *

361 TGGCTTGGCT TCTGTAAATC ATGCAGAGAC TAAGCCATTT TTTATTTGGT AATTATAAGA

421 AGGAGTTTGC CTTTATAGAG AACGGGAAAA CATAGAGTGG AATTCATAGA AAGAGGGCGT

481 GAAATATGGA CCAACAAAAA AAGATTCAAA TTTTAAAGGA CTTGGTAAAT ATTGATTCGA

541 CTAATGGGCA TGAAGAACAA GTTGCGAACT ATTTGCAAAA GTTGTTAGCT GAACATGGTA

601 TTGAGTCCGA AAAGGTACAA TACGACCTAG ACAGAGCTAG CCTAGTAAGC GAAATTGGTT

661 CCAGTAACGA GAA
```

Nucleotide sequence of *L. monocytogenes* Δ*inlB/C/GHE*, BC16 (S36)

Length of the DNA fragment sequenced: 673 bp. Genes *inlG* and *inlE* fused in frame to each other. Resulting mutant gene encodes a 29-amino acid residual peptide.

```
  1 AGCACCGCTT TGGCGGGGCT GTTTTTTTAC GCTACTTCCC ACAAGAAACC AATTCTGCAA

 61 TCGCTGAACG AAAAAGTTCA ATTAATTGTC ACATCTTTCG TGTAGAAGGG TCTAATGCGA

121 GAAAAAATGG CGGGCATCTT CTGTTATAAT TATTCATCAA AGACTAGTAA TATTTTACGC

181 CAAGTCATCC AAGATAGTGC AGATCCAAGG TGTATTTAAT GATGAATTAT CTACTCAGTC

241 AAATTATAAG GAGACGATAA AATGAAACAG AGAAAAACCT CAGTACTACA TGTTTTACTT
                                  M   K   Q   R   K   T   S   V   L   H   V   L   L

301 GTAGTGACAG CTATCCCGGG TTTATTCCGC AAAAAACGTC AAACAAAATA AACCAATCAA
       V   V   T   A   I   P   G   L   F   R   K   K   R   Q   T   K   *

361 TGGCTTGGCT TCTGTAAATC ATGCAGAGAC TAAGCCATTT TTTATTTGGT AATTATAAGA

421 AGGAGTTTGC CTTTATAGAG AACGGGGAAA CATAGAGTGG AATTCATAGA AAGAGGGCGT

481 GAAATATGGA CCAACAAAAA AAGATTCAAA TTTTAAAGGA CTTGGTAAAT ATTGATTCGA

541 CTAATGGGCA TGAAGAACAA GTTGCGAACT ATTTGCAAAA GTTGTTAGCT GAACATGGTA

601 TTGAGTCCGA AAAGGTACAA TACGACCTAG ACAGAGCTAG CCTAGTAAGC GAAATTGGTT

661 CCAGTAACGA GAA
```

Nucleotide sequence of *L. monocytogenes* ∆*inlA/C* 3-6 (S71)

Length of the DNA fragment sequenced: 538 bp. Nucleotide sequence as expected. In-frame deletion of most *inlC* gene. Resulting mutant gene codes for a 80-amino acid peptide.

```
  1 TAGAACATGT TTTGAAAAAT TTACTGATTT TCGATTATTA TTAACGCTTG TTAATTTAAA

 61 CATCTCTTAT TTTTGCTAAC ATATAAGTAT ACAAAGGGAC ATAAAAAGGT TAACAGCGTT

121 TGTTAAATAG GAAGTATATG AAAATCCTCT TTTGTGTTTC TAAATTTATT TTTAAGGAGT

181 GGAGAATGTT GAAAAAAAAT AATTGGTTAC AAAATGCAGT AATAGCAATG CTAGTGTTAA
             M    L   K   K   N    N   W   L    Q   N   A    V   I   A   M    L   V   L

241 TTGTAGGTCT GTGCATTAAT ATGGGTTCTG GAACAAAAGT ACAACCCGGG AGTTATGTAG
     I   V   G    L   C   I   N    M   G   S    G   T   K    V   Q   P   G    S   Y   V

301 ATGGTTGTGT CCTGTGGGAA TTGCCAGTTT ATACAGATGA AGTAAGCTAT AAGTTTAGCG
     D   G   C    V   L   W   E    L   P   V    Y   T   D    E   V   S   Y    K   F   S

361 AATATATAAA CGTTGGGGAG ACTGAGGCTA TATTTGATGG AACAGTTACA CAACCTATCA
     E   Y   I    N   V   G   E    T   E   A    I   F   D    G   T   V   T    Q   P   I

421 AGAATTAGGA CTTGTGCACA CCTGTATACT TTGAGCTCTC GTATAATCAC GAGAGCTTTT
     K   N   *

481 TAAATATGTA AGTCTTAATT ATCTCTTGAC AAAAAGAACG TTTATTCGTA TAAGGTTA
```

Nucleotide sequence of *L. monocytogenes* ∆*inlA/C* 4-8 (S73)

Length of the DNA fragment sequenced: 553 bp. In-frame deletion of most *inlC* gene.
Resulting mutant gene codes for a 80-amino acid peptide.

```
  1 GGAAGGTAGA ACATGTTTTG AAAAATTTAC TGATTTTCGA TTATTATTAA CGCTTGTTAA


 61 TTTAAACATC TCTTATTTTT GCTAACATAT AAGTATACAA AGGGACATAA AAAGGTTAAC


121 AGCGTTTGTT AAATAGGAAG TATATGAAAA TCCTCTTTTG TGTTTCTAAA TTTATTTTTA


181 AGGAGTGGAG AATGTTGAAA AAAAATAATT GGTTACAAAA TGCAGTAATA GCAATGCTAG
              M   L   K    K   N   N    W   L   Q    N   A   V   I    A   M   L

241 TGTTAATTGT AGGTCTGTGC ATTAATATGG GTTCTGGAAC AAAAGTACAA CCCGGGAGTT
     V   L   I    V   G   L   C    I   N   M    G   S   G    T   K   V   Q    P   G   S

301 ATGTAGATGG TTGTGTCCTG TGGGAATTGC CAGTTTATAC AGATGAAGTA AGCTATAAGT
     Y   V   D    G   C   V   L    W   E   L    P   V   Y    T   D   E   V    S   Y   K

361 TTAGCGAATA TATAAACGTT GGGGAGACTG AGGCTATATT TGATGGAACA GTTACACAAC
     F   S   E    Y   I   N   V    G   E   T    E   A   I    F   D   G   T    V   T   Q

421 CTATCAAGAA TTAGGACTTG TGCACACCTG TATACTTTGA GCTCTCGTAT AATCACGAGA
     P   I   K    N   *

481 GCTTTTTAAA TATGTAAGTC TTAATTATCT CTTGACAAAA AGAACGTTTA TTCGTATAAG


541 GTTACCAAGA GAT
```

Nucleotide sequence of *L. monocytogenes* Δ*inlA/C/GHE* 3-5 (S75)

Length of the DNA fragment sequenced: 538 bp. In-frame deletion of most *inlC* gene.
Resulting mutant gene codes for a 80-amino acid peptide.

```
  1 TAGAACATGT TTTGAAAAAT TTACTGATTT TCGATTATTA TTAACGCTTG TTAATTTAAA


 61 CATCTCTTAT TTTTGCTAAC ATATAAGTAT ACAAAGGGAC ATAAAAAGGT TAACAGCGTT


121 TGTTAAATAG GAAGTATATG AAAATCCTCT TTTGTGTTTC TAAATTTATT TTTAAGGAGT


181 GGAGAATGTT GAAAAAAAT AATTGGTTAC AAAATGCAGT AATAGCAATG CTAGTGTTAA
            M   L  K  K  N   N  W  L   Q  N  A   V  I  A  M   L  V  L

241 TTGTAGGTCT GTGCATTAAT ATGGGTTCTG GAACAAAAGT ACAACCCGGG AGTTATGTAG
     I  V  G   L  C  I  N   M  G  S   G  T  K   V  Q  P  G   S  Y  V

301 ATGGTTGTGT CCTGTGGGAA TTGCCAGTTT ATACAGATGA AGTAAGCTAT AAGTTTAGCG
     D  G  C   V  L  W  E   L  P  V   Y  T  D   E  V  S  Y   K  F  S

361 AATATATAAA CGTTGGGGAG ACTGAGGCTA TATTTGATGG AACAGTTACA CAACCTATCA
     E  Y  I   N  V  G  E   T  E  A   I  F  D   G  T  V  T   Q  P  I

421 AGAATTAGGA CTTGTGCACA CCTGTATACT TTGAGCTCTC GTATAATCAC GAGAGCTTTT
     K  N  *

481 TAAATATGTA AGTCTTAATT ATCTCTTGAC AAAAAGAACG TTTATTCGTA TAAGGTTA
```

Nucleotide sequence of *L. monocytogenes* Δ*inlA/C/GHE* 4-14 (S76)

Length of the DNA fragment sequenced: 559 bp. In-frame deletion of most *inlC* gene. Resulting mutant gene codes for a 80-amino acid peptide.

```
  1 CAACAAGGAA GGTAGAACAT GTTTTGAAAA ATTTACTGAT TTTCGATTAT TATTAACGCT


 61 TGTTAATTTA AACATCTCTT ATTTTTGCTA ACATATAAGT ATACAAAGGG ACATAAAAAG


121 GTTAACAGCG TTTGTTAAAT AGGAAGTATA TGAAAATCCT CTTTTGTGTT TCTAAATTTA


181 TTTTTAAGGA GTGGAGAATG TTGAAAAAAA ATAATTGGTT ACAAAATGCA GTAATAGCAA
                         M   L   K   K   N   N   W   L   Q   N   A   V   I   A

241 TGCTAGTGTT AATTGTAGGT CTGTGCATTA ATATGGGTTC TGGAACAAAA GTACAACCCG
     M   L   V   L   I   V   G   L   C   I   N   M   G   S   G   T   K   V   Q   P

301 GGAGTTATGT AGATGGTTGT GTCCTGTGGG AATTGCCAGT TTATACAGAT GAAGTAAGCT
      G   S   Y   V   D   G   C   V   L   W   E   L   P   V   Y   T   D   E   V   S

361 ATAAGTTTAG CGAATATATA AACGTTGGGG AGACTGAGGC TATATTTGAT GGAACAGTTG
      Y   K   F   S   E   Y   I   N   V   G   E   T   E   A   I   F   D   G   T   V

421 CACAACCTAT CAAGAATTAG GACTTGTGCA CACCTGTATA CTTTGAGCTC TCGTATAATC
      A   Q   P   I   K   N   *

481 ACGAGAGCTT TTTAAATATG TAAGTCTTAA TTATCTCTTG ACAAAAAGAA CGTTTATTCG


541 TATAAGGTTA CCAAGAGAT
```

B.3 Nucleotide sequence of the *inlF* gene of *L. monocytogenes* EGD

```
   1   AGCTGGCTTG ACAAAATTGG ACAATATAGC AGCATATTCG AATAAAATCA

  51   CTGATATTAC TCCTGTGACC AATTTAACAA GACTCCAGTA TTTGGATTTA

 101   GGTAGTAATG AAATCACTGA TTTAAGTCCT GTGGCTAATC TGCAAAAATT

 151   AACCTCGCTA CATCTTGCAA ACAACCAGAT TACTAATATT AGTATGCTTG

 201   AAGATTTAAC AAATTTAACT TCGTTGGGTT TACAAAACAA TAAAATTAGT

 251   GATATATCCG TTTTGAAAAA TCTAACCCAT GTGACTTATT TGCAGCTGGG

 301   GTATAACCAA ATAGTGGATG TGAAAATAAT CGGAGGACTA ACTAATTTAA

 351   CAAGTTTGCA GTTAACACAA AACCATATTA CTGACATAAG TCCTTTAGCC

 401   AACTTAACCA AAATACAATA CTCTGACTTC TCTAATCAGA TGATAACAAA

 451   TCTAGAACGT AATTTTTCGA AGACACTCTC CGTTCCGAAC AATATAACTA

 501   GCATAGATGG AACGCTAATT GCGCCTGAAA CGATTAGCAA TAATGGAACC

 551   TACGACGCAC CGAACTTGAA GTGGTCTTTA CCGAACTATT TACCAGAAGT

 601   TAAATATACG TTCAGCCAAA AAATACCGAT TGGGACAGGC ACAAGTAATT

 651   ATAGTGGCTT CATAACACAA CCGTTAAAAG AATTACTAGA TTACAAAGTC

 701   ACATTTAATG TAGAAGGTAA TACAAGTGAA GTAGAGACTG TAACAGAAGA

 751   AAATCTCATT CCAGAACCTA CGAGCCCAAC CAAACAAGGT TATACATTTG

 801   ATGGTTGGTA CGACGCAGAA ACAGGCGGAA CAAAATGGGA CTTCACAACC

 851   GGGCAAATGC CTGCAAATGA CCTCACACTA TATGCCCATT TTTCCGTAAA

 901   TAGCTACCAA GCAAATTTTG ATATAGACGG TGTGGTAACG AATGAAGCGG

 951   TAGTATACGA TACCTTACTC AATGAACCGA CCACTCCAAC CAAACAAGGC

1001   TATACATTTG ATGGCTGGTA TGACGCAGAA ACAGGCGGTA ATAAGTGGGA

1051   TTTCAAAACA ATGAAAATGC CCGCGAATGA TGTTGCTTTT TATGCACATT

1101   TTACTATCAA CAACTATCAA GCAAATTTTG ATATAGATGG TGAGGTAAAG

1151   AATGAAACGA TAGCATACGA TACCTTACTC AATGAACCGA CCACTCCAAC

1201   C
```

Sequence alignment of internalins

```
        1                                                        50
InlC2   MKKRWNSVFK LVLMVTAILG LSLYVTTSQG VEV..RAESI TQPTAINVIF
 InlH   MKKRWNSVFK LVLMVTAILG LSLYVTTSQG VEV..RAESI TQPTAINVIF
 InlD   .MKRNKTALR ILVTLAVVMA ISFWVGTS.S KEV..QAAEI GQPTPINEIF
 InlE   .MKRNKTALR ILVTLAVVLA ITFWVGMS.S KEV..QAAVI EHPTPINEIF
 InlG   MKQRKTSVLH VLLVVTAILG ISLWVNASHG MKA..QAESI AQPAPINEIF
 InlA   MRKKRYVWLK SILVAILVFG SGVWINTSNG TNA..QAATI TQDTPINQIF
 InlF   .MKSKNNYFK QIITIMTVVS LLIMVLGIQG NNDVKAATQV APPASINQIF
 InlB   VKEKHNPRRK YCLISGLAII FSLWIIIGNG AKV..QAETI TVSTPIKQIF

        51                                                       100
InlC2   PDPALANAIK IAAGKSNVTD TVTQADLDGI TTLSAFGTGV TTIEGVQYLN
 InlH   PDPALANAIK IAAGKSNVTD TVTQADLDGI TTLSAFGTGV TTIEGVQYLN
 InlD   TDENLANAIK TTLSKPSTAS AVSQVELDSV RDVTAESSNI ASLEGVQYLN
 InlE   TDPVLTDNVK TLLGKADVTD EVTQTDLDSV THLSAKSAGI TTIEGLQYLT
 InlG   TDPALADEVK TELGKTSVTD EVTQTDLNQI TKLEADDKGI NSIEGIQYLT
 InlA   TDAALAEKMK TVLGKTNVTD TVSQTDLDQV TTLQADRLGI KSIDGLEYLN
 InlF   PDADLAEGIR AELQKSSVTD VVTKEELESI SQLSVYAKKI ASIEGLEYLT
 InlB   PDDAFAETIK DNLKKKSVTD AVTQNELNSI DQIIANNSDI KSVQGIQYLP

        101                                                      150
InlC2   NLIGLELKDN QITDLTPLKN LTKITELELS GNPLKNVSAI AGLQSIKTLD
 InlH   NLIGLELKDN QITDLTPLKN LTKITELELS GNPLKNVSAI AGLQSIKTLD
 InlD   NLDTLVLNNN KITDLNPLAG LTKLSILEAS NNQLSDISAL SNVTNLHQLR
 InlE   NLSELELIDN QVTDLNPLKN LTKITELRLS GNPLKDVSAL AGLKNLKTMD
 InlG   NLNMLGVSSN QITNITPLAN LT........ .......... ..........
 InlA   NLTQINFSNN QLTDITPLKD LTKLVDILMN NNQIADITPL ANLTNLTGLT
 InlF   NLKFLNLNGN QITDLSPLSN LTKLTEIYIG DNKISDISPL QNLTNVTDLY
 InlB   NVTKLFLNGN KLTDIKPLTN LKNLGWLFLD ENKIKDLSSL KDLKKLKSLS

        151                                                      200
InlC2   LTSTQITDVT PLAGLSNLQV LYLDLN.QIT NISPLAGLTN LQYLSIG...
 InlH   LTSTQITDVT PLAGLSNLQV LYLDLN.QIT NISPLAGLTN LQYLSIG...
 InlD   LDGNQIKQLN GVSNLINLET IELSNN.QIT AISPVSGLKN LVGLGID...
 InlE   LIYTDITDVT PLAGLSNLQV LNLDIN.QIT DITPLAGLSN LQFLSFG...
 InlG   .......... ......NLDS LYLGDN.KIS DVTPLSGLTQ LTFVQLS...
 InlA   LFNNQITDID PLKNLTNLNR LELSSN.TIS DISALSGLTN LQQLSFGNQV
 InlF   LVDNDISDLR PLANLTQMYS LRLGGNSNIS DLNPVRNMTR LNNLEVTGSI
 InlB   LEHNGISDIN GLVHLPQLES LYLGNN.KIT DITVLSRLTK LDTLSLE...

        201                                                      250
```

```
InlC2   .......... .......... .......... .......... ..........
 InlH   .......... .......... .......... .......... ..........
 InlD   .......... .......... .......... .......... ..........
 InlE   .......... .......... .......... .......... ..........
 InlG   .......... .......... .......... .......... ..........
 InlA   TDLKPLANLT TLERLDISSN KVSDISVLAK LTNLESLIAT NNQISDITPL
 InlF   .......... .......... .......... .......... ...LKDLTPL
 InlB   .......... .......... .......... .......... ..........


        251                                              300
InlC2   .......... .......... .......... ....NAQVSD LTPLANLSKL
 InlH   .......... .......... .......... ....NAQVSD LTPLANLSKL
 InlD   .......... .......... .......... ....NNKISD LSPISGLSKL
 InlE   .......... .......... .......... ....STQVSD LTPLANLSKL
 InlG   .......... .......... .......... ....INQIKD VTPLANLTKL
 InlA   GILTNLDELS LNGNQLKDIG TLASLTNLTD LDLANNQISN LAPLSGLTKL
 InlF   ADVTSLTRLT LSDNQIEDLS PLAGLTKLDN IAAYSNKITD ITPVTNLTRL
 InlB   .......... .......... .......... ....DNQISD IVPLAGLTKL


        301                                              350
InlC2   TTLKADDNKI SDISPLASLP NLIEVHLKNN QISDVSPLA. ..........
 InlH   TTLKADDNKI SDISPLASLP NLIEVHLKNN QISDVSPLA. ..........
 InlD   NHLTADSNQI SDLRPLSNLA AMEVMRLDGN QISDVTPIAN LANLNYVFLA
 InlE   TTLNAMNSKV SDVSPLTGLS NLTEVYLEEN QISDVSPLAK L.........
 InlG   NYLDLRENQI SDASPLVNMT DLTVLHLEKQ Q......... ..........
 InlA   TELKLGANQI SNISPLAGLT ALTNLELNEN QLEDISPISN LKNLTYLTLY
 InlF   QYLDLGSNEI TDLSPVANLQ KLTSLHLANN QITNISMLED LTNLTSLGLQ
 InlB   QNLYLSKNHI SDLRALAGLK NLDVLEL... .......... ..........


        351                                              400
InlC2   .......... ....NTSNLF I......... .......... ..........
 InlH   .......... ....NTSNLF I......... .......... ..........
 InlD   ENQISDISSL QPLFNSPNFF G......... .......... ..........
 InlE   .......... .....PNLS I......... .......... ..........
 InlG   .......... .......... .......... .......... ..........
 InlA   FNNISDISPV SSLTKLQRLF FYNNKVSDVS SLANLTNINW LSAGHNQISD
 InlF   NNKISDISVL KNLTHVTYLQ LGYNQIVDVK IIGGLTNLTS LQLTQNHITD
 InlB   .......... .......... .......... .......... ..........


        401                                              450
InlC2   .......... ..VTLTNQTI TNQPVFYQNN LVVPNIVKGP SGAPIAPATI
 InlH   .......... ..VTLTNQTI TNQPVFYQNN LVVPNVVKGP SGAPIAPATI
 InlD   .......... ..ITLDNQKI TSEPVLYQQE LVVPNNIKDE MGALIAPATI
 InlE   .......... ..VTLTNQTI TNQPVFYQNK PIVPNVVTGL SGELIAPDTI
 InlG   .......... .........I TAAPVVYQTN LVAPDILKNA YGEVVPPTTI
 InlA   LTPLANLTRI TQLGLNDQAW TNAPVNYKAN VSIPNTVKNV TGALIAPATI
 InlF   ISPLANLTKI QYSDFSNQMI TNLERNFSKT LSVPNNITSI DGTLIAPETI
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```
InlB    .......... .....FSQEC LNKPINHQSN LVVPNTVKNT DGSLVTPEII


        451                                                   500
InlC2   SDNGTYASPN LTWNLTSFIN NVSYTFNQSV TF.KNTTVPF SGTVTQPLTE
 InlH   SDNGTYASPN LTWNLTSFIN NVSYTFNQSV TF.KNTTVPF SGTVTQPLTE
 InlD   SDNGVYASPN INWNLPNYTN QVSYTFNKQL AY.....GSF SGTVTQPLHN
 InlE   SDNGTYTSPN LTWDLNSFIN SVSYTFNQSV TF.KNTTAPF SGTVTQPLTE
 InlG   SNNGTFASPN ITWNLDSFTS EVSYDFNQKI TLGDNGKVTF AGTVVQPIVE
 InlA   SDGGSYAEPD ITWNLPSYTN EVSYTFSQPV TIGKGTT.TF SGTVTQPLKA
 InlF   SNNGTYDAPN LKWSLPNYLP EVKYTFSQKI PIGTGTS.NY SGFITQPLKE
 InlB   SDDGDYEKPN VKWHLPEFTN EVSFIFYQPV TIGK.AKARF HGRVTQPLKE


        501                                                   550
InlC2   .......... .......... .......... .......... ..........
 InlH   .......... .......... .......... .......... ..........
 InlD   .......... .......... .......... .......... ..........
 InlE   .......... .......... .......... .......... ..........
 InlG   .......... .......... .......... .......... ..........
 InlA   IFNAKFHVDG KETTKEVEA. ..GNLLTEPA KPVKEGHTFV GWFDAQTGGT
 InlF   LLDYKVTFNV EGNTSEVETV TEENLIPEPT SPTKQGYTFD GWYDAETGGT
 InlB   V......... .......... .......... .......... ..........


        551                                                   600
InlC2   .......... .......... A...YTAVFD VDGKQTSVTV GANELIKEPT
 InlH   .......... .......... A...YTAVFD VDGKQTSVTV GANELIKEPT
 InlD   .......... .......... A...YTATFD VDGVKTNEAV EETKLLQEPI
 InlE   .......... .......... V...YAVVFD VDGEQTSAMV GVNELINEPT
 InlG   .......... .......... APVNYITTFD VDGTTTTENV VVDTLITEPA
 InlA   KWNFSTDKMP TNDINLYAQF SINSYTATFE NDGVTTSQTV DYQGLLQEPT
 InlF   KWDFTTGQMP ANDLTLYAHF SVNSYQANFD IDGVVTNEAV VYDALLNEPT
 InlB   .......... .......... ....YTVSYD VDGTVIKTKV EAGTRITAPK


        601                                                   650
InlC2   APTKEGYTFT GWYDAKTGGN KWDFGVDKMP AENITLYAQF TINSYTASFD
 InlH   APTKEGYTFT GWYDAKTGGT KWDFATDKMP AEDITLYAQF TINSYTATFD
 InlD   APTKEGYTFT GWYDAKTGGN KWDFATDKMP AEDITLYAQF TINSYTATFD
 InlE   APAKEGYIFD GWYDAKTDGN KWDFGIDKMP ASDITLYAKF TEN.......
 InlG   EPTKEGYTFS GWYDAETGGN EWDFAVDKMP ATNMTLYAQF TINSYTATFD
 InlA   PPTKEGYTFK GWYDAKTGGD KWDFATSKMP AKNITLYAQY SANSYTATFD
 InlF   TPTKQGYTFD GWYDAETGGN KWDFKTMKMP ANDVAFYAHF TINNYQANFD
 InlB   PPTKQGYVFK GWYTEKNGGH EWNFNTDYMS GNDFTLYAVF KAETTEKTVN


        651                                                   700
InlC2   N......... DGKLTTQKVT YQSLLEEPAA PTKTGYTFKG WYDAKTGGTK
 InlH   I......... DGKLTTQKVT YQSLLEEPVA PTKDGYTFTG WYDAKTGGTK
 InlD   I......... DGKLTTQKVT YQSLLEEPVA PTKDGYTFTG WYDAKTGGTK
 InlE   .......... .......... .....EEPNA S......... ..........
```

```
InlG  V.........  DGETTNQKVD  YQALLQEPTA  PTKDGYTFVG  WYDAKTGGTE
InlA  V.........  DGKSTTQAVD  YQGLLKEPKA  PTKAGYTFKG  WYDEKTDGKK
InlF  I.........  DGEVKNETIA  YDTLLNEPTT  PTKQGYTFDG  WYDAETGGTK
InlB  LTRYVKYIRG  NAGIYKLPRE  DNSLKQGTLA  SHRCKALTVD  REARNGGKLW


      701                                                      750
InlC2 WDFATGKMPA  GNITLYAQFT  KNDSPNP...  ..........  ..........
InlH  WDFATGKMPA  GNITLYAQFT  KNDNPNP...  ..........  ..........
InlD  WDFATGKMPA  GNITLYAQFT  KNDNPNP...  ..........  ..........
InlE  ........SP  INVEPNDNNS  DNAEPNA...  ..........  ..........
InlG  WDFATSKMPT  SDITLYARFT  KNPSSDN...  ..........  ..........
InlA  WDFATDKMPA  NDITLYAQFT  KNPVAPP...  ..........  ..........
InlF  WDFKTKEMPA  NDVTLYAHFT  INNYQANFDI  DGAVTEEVVN  YDALIPEPTS
InlB  YRLKNIGWTK  AENLSLDRYD  KMEYDKGVTA  YARVRNASGN  SVWTKPYNTA


      751                                                      800
InlC2 ..........  ..........  ..........  ........N  DPTPNTPTGN
InlH  ..........  ..........  ..........  ........D  DPTTNTPTGN
InlD  ..........  ..........  ..........  ........D  DPTTNTPTGN
InlE  ..........  ..........  ..........  ........S  SSINVQENGT
InlG  ..........  ..........  ..........  ........S  QTAPGKDDKN
InlA  ..........  ..........  ..........  ........T  TGGNTPPTTN
InlF  PSKTGFTLEG  WYDAEVGGTK  WDFKTMKMPA  NDITLYAHFS  KETPIIPSPD
InlB  GAKHVNKLSV  YQGKNMRILR  EAKTPITTWY  QFSIGGKVIG  WVDTRALNTF


      801                                                      850
InlC2 G.DGTSNPSN  ..........  SGG...NTT.  ..........  ....LPTAGD
InlH  G.DGTSNPSN  ..........  SGG...NTT.  ..........  ....LPTAGD
InlD  G.DGTSNPSN  ..........  SGG...NTT.  ..........  ....LPTAGD
InlE  N.EGINNLNS  ..........  SGEDKVNIK.  ..........  ....LPITGD
InlG  DKDKLTIKAN  ..........  DSADATSTK.  ..........  ....LPKTSD
InlA  NGGNTTPPSA  NIPGSDTSNT  STGNSASTTS  TMNAYDPYNS  KEASLPTTGD
InlF  EGLDSDSTNG  PITINEPSAT  STPSQNNNIT  VTAGENTTEL  ATAKLPKTGD
InlB  YKQSMEKPTR  LTRYVSANKA  GESYYKVPVA  DNPVKRGTLA  KYKNQKLIVD


      851                        883
InlC2 ENTMLPIFIG  VFLLGTATLI  LRKTIKVK*.  ...
InlH  ENTMLPIFIG  VFLLGTATLI  LRKTIKVK*.  ...
InlD  ENTMLPIFIG  VFLLGTATLI  LRKTIKVK*.  ...
InlE  ELNVLPIFVG  AVLIGIGLVL  FRKKRQTK*.  ...
InlG  DSSMIPTILG  TLFIGGAILI  LRKKTTNI*.  ...
InlA  SDNALYLLLG  LLAVGTAMAL  TKKARASK*.  ...
InlF  NAPWKTLFAG  ILLSSSAFYI  WRKKA*....  ...
InlB  CQATIEGQLW  YRIRTSSTFI  GWTKAANLRA  QK*
```

Abbreviations

A .... adenine/adenosine, alanine
Amp .... ampicillin
App. .... appendix
ATCC . American Type Culture Collection
ATP .... adenosine triphosphate
BHI .... brain heart infusion
bp .... base pairs
C .... cytosine/cytidine, carboxyl
$CaCl_2$ .... calcium chloride
cDNA .... complementary DNA
CFU .... colony forming units
Chap. .... chapter
°C .... degrees Celsius
D .... aspartic acid
Da .... Dalton
deion. .... deionized
DNA .... deoxyribonucleic acid
DNase .... deoxyribonuclease
dNTPs . deoxyribonucleoside triphosphate
dsDNA .... double stranded DNA
E. .... *Escherichia*
ELISA ... enzyme-linked immunosorbent
assay
Em .... erythromycin
EtOH .... ethanol
FACS ... fluorescence-activated cell sorter
FCS .... fetal calf serum
Fig. .... figure
g .... gram
G .... guanine/guanosine, glycine
gfp, GFP .... green fluorescent protein
h .... hour
HBMEC ..... human brain microvascular
endothelial cells
I .... isoleucine
inl .... internalin
i. v. .... intravenous
K .... lysine

kb .... kilo base
kDa .... kilo Dalton
KE .... Klett Einheiten, Klett units
Km .... kanamycin
l .... liter
L .... leucine
L. .... *Listeria*
LD_{50} .. lethal dose for 50% of the animals
LRRs .... leucine-rich repeats
M molar, DNA molecular weight standard
MEM .... minimal essential medium
$MgCl_2$ .... magnesium chloride
$MgSO_4$ .... magnesium sulfate
min .... minute
ml .... milliliter
mm .... millimeter
mM .... millimolar
mRNA .... messenger RNA
MOI .... multiplicity of infection
μl .... microliter
μg .... microgram
N ..... asparagine, any nucleotide, amino
N. .... *Neisseria*
NaCl .... sodium chloride
NCTC ...... National Collection of Type
Culture
ng .... nanogram
nm .... nanometer
OD .... optical density
ORF .... open reading frame
P .... promoter, proline
PBS .... phosphate-buffered saline
PCR .... polymerase chain reaction
p. i. .... post-infection
PI .... propidium iodide
PC-PLC ..... phosphatidylcholin-specific
phospholipase C
PI-PLC ..... phosphatidylinositol-specific

phospholipase C

R .... resistance

RBS .... ribosome binding site

RNA .... ribonucleic acid

RNase .... ribonuclease

RPM .... revolutions per minute

RT .... room temperature, reverse transcription

RT-PCR . reverse transcription polymerase chain reaction

S .... serine

S. Shigella, *Staphylococcus*, *Streptococcus*

SDS .... sodium dodecyl sulfate

sec .... second

SLCC   Special *Listeria* Culture Collection

ssp. .... species

ssDNA .... single stranded DNA

ster. .... sterile

sv .... serovar

T .... thymine/thymidine, threonine

Tab. .... table

Tc .... tetracycline

ts .... temperature sensitive

U .... units

UV .... ultraviolet

vol,v .... volume

W .... trytophan

WT .... wild type

X .... any amino acid

Y. .... *Yersinia*

YT .... yeast tryptone

Publications

D. Raffelsbauer, A. Bubert, F. Engelbrecht, J. Scheinpflug, A. Simm, J. Hess, S. H. E. Kaufmann and W. Goebel (1998). The gene cluster *inlC2DE* of *Listeria monocytogenes* contains additional new internalin genes and is important for virulence in mice. *Mol. Gen. Genet.* 260:144–158.

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[1]Both authors contributed equally to this paper.

Curriculum Vitæ

Name:	Diana Raffelsbauer
Date of birth:	July, 1 1968
Place of birth:	Fortaleza – Brazil
Citizenship:	Brazilian
Address:	Heigern 11
	97286 Winterhausen
	Germany
	✆ +49-9333-1758
	✉ diana@infobio.de

School

Feb. 1975 – Dez. 1978:   Elementary School in Fortaleza, Brazil.

Feb. 1979 – Dez. 1985:   High School in Fortaleza, Brazil.
Certificate: High school diploma.

Training

Aug. 1987 – July 1988:   Training as bank employee at Banco do Nordeste do Brasil, Fortaleza, Brazil.

University

Studies in Biology

Aug. 1986 – July 1987 and
Aug. 1989 – July 1990:   Universidade Federal do Ceará, Fortaleza, Brazil.

Nov. 1991 – Aug. 1997:   Bayerische Julius–Maximilians-Universität Würzburg, Germany.
Certificate: Diplom–Biologin Univ.
Grade: "Sehr gut" (Excellent).

PhD Studies in Biology

Since September 1997:   Julius-Maximilians-Universität Würzburg, Germany, Department of Microbiology.

Employment and Scholarships

Aug. 1988 – Nov. 1990:   Bank employee at Banco do Nordeste do Brasil, offices of Rio de Janeiro, ETENE and Fortaleza-Centro, Brazil.

Jan. – March 1990:   German course at the Institute Goethe in Rothenburg o. d. T. Scholarship from the Institute Goethe of Munich, Germany.

April 1994 – May 1997:   Scholarship from the "Begabtenförderung der Friedrich-Naumann-Stiftung", Potsdam, Germany.

Aug. 1994 – Jan. 1995:   Study as graduate student in Biology at the State University of New York (SUNY) – Albany, USA . Scholarship from SUNY–Albany.

June 1996 – Aug. 1997:   Master's thesis on "'Internaline von *Listeria monocytogenes* und *Listeria ivanovii*" at the Department of Microbiology of the University of Würzburg, Germany. Scholarship from the Friedrich-Naumann-Stiftung.

Sept. 1996 – April 1997:   Employee at the Departments of Microbiology and Genetics of the University of Würzburg, Germany.

Sept. 1997 – Feb. 2001:   Employee at the Department of Microbiology of the University of Würzburg, Germany.
PhD thesis on "Identification and characterization of the *inlGHE* of *Listeria monocytogenes*". Financial support from the "Deutsche Forschungsgemeinschaft", SFB 479-B1.

Lebenslauf

Name:	Diana Raffelsbauer
Geburtsdatum:	1. Juli 1968
Geburtsort:	Fortaleza – Brasilien
Staatsangehörigkeit:	Brasilianisch
Anschrift:	Heigern 11
	97286 Winterhausen
	☎ +49-9333-1758
	✉ diana@infobio.de

Schulbildung

Feb. 1975 – Dez. 1978:   Grundschule in Fortaleza, Brasilien.

Feb. 1979 – Dez. 1985:   Gymnasium in Fortaleza, Brasilien.
Abschluß: Certificado de Ensino de 2º Grau
(Allgemeine Hochschulreife).

Berufsausbildung

Aug. 1987 – Juli 1988:   Ausbildung zu Bankkauffrau bei Banco do Nordeste do Brasil, Fortaleza, Brasilien.

Hochschulbildung

Studium der Biologie

Aug. 1986 – Juli 1987 und
Aug. 1989 – Juli 1990:   Universidade Federal do Ceará, Fortaleza, Brasilien.

Nov. 1991 – Aug. 1997:   Bayerische Julius–Maximilians-Universität Würzburg.
Abschluß: Diplom–Biologin Univ.
Gesamtnote: Sehr gut.

Promotionsstudium in Biologie

Seit September 1997:   Julius-Maximilians-Universität Würzburg, Lehrstuhl für Mikrobiologie.

Berufstätigkeit und Stipendien

Aug. 1988 – Nov. 1990:   Bankkauffrau bei Banco do Nordeste do Brasil, Filiale Rio de Janeiro, ETENE und Fortaleza-Centro, Brasilien.

Jan. – März 1990:   Deutschkurs am Goethe-Institut in Rothenburg o. d. T. Stipendium vom Goethe-Institut München.

April 1994 – Mai 1997:   Stipendiatin der Begabtenförderung der Friedrich-Naumann-Stiftung, Potsdam.

Aug. 1994 – Jan. 1995:   Studium an der State University of New York (SUNY) – Albany, USA als "'Graduate Student'" in Biologie. Stipendium von SUNY–Albany.

Juni 1996 – Aug. 1997:   Diplomarbeit über "Internaline von *Listeria monocytogenes* und *Listeria ivanovii*" am Lehrstuhl für Mikrobiologie der Universität Würzburg. Stipendium der Friedrich-Naumann-Stiftung.

Sept. 1996 – April 1997:   Studentische Hilfskraft an den Lehrstühlen für Mikrobiologie und Genetik der Universität Würzburg.

Sept. 1997 – Feb. 2001:   Wissenschaftliche Mitarbeiterin des Lehrstuhls für Mikrobiologie der Universität Würzburg.
Doktorarbeit "Identification and characterization of the *inlGHE* gene cluster of *Listeria monocytogenes*". Finanzielle Unterstützung der Deutschen Forschungsgemeinschaft, SFB 479-B1.

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