Listeria monocytogenes - Strain Diversity Demonstrated by Genotyping

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Abstract

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The bacterium *Listeria monocytogenes* can cause the disease listeriosis in both humans and animals. For humans, the infection is mainly foodborne. Since the bacterium is able to grow even at refrigerator temperature, a food product may constitute a possible vehicle of infection although stored in cold environment. Meningoencephalitis and septicaemia are clinical manifestations of listeriosis. Infection in a pregnant woman may lead to abortion of the foetus or the birth of a seriously ill child. Risk groups for *L. monocytogenes* infection are immunocompromised persons, the elderly, pregnant women and infants.
Different methods for visualising the genetic diversity among strains of *L. monocytogenes* were used in this thesis.

L. monocytogenes can be established in food processing plants for long periods. Investigation of genetic diversity among strains isolated from a dairy during several years by pulsed-field gel electrophoresis (PFGE) showed indistinguishable restriction patterns. This indicated a common source for the *L. monocytogenes* contamination. The PFGE method was also used to investigate the diversity among strains isolated from faeces from clinically healthy cows.

Polymerase chain reaction and restriction enzyme analysis (PCR-REA) of a segment of a virulence gene region could divide strains of serovar 1/2a into two groups. Strain diversity was also visualised by sequencing. Both conventional sequencing of 1500 bp of the virulence gene *inlB* and the sequencing-by-synthesis method pyrosequencing of two positions of this gene, grouped *L. monocytogenes* strains in a similar way. Strains of serovars 1/2a and 1/2c formed one group, strains of serovar 1/2b and 3b another and strains of serovar 4b were divided into two groups. Based on nucleotide polymorphisms in the *inlB* gene the strain diversity within serovar 4b was more distinct than within the other investigated serovars.

Keywords: *Listeria monocytogenes*, foodborne pathogen, contamination, genetic diversity, typing, pulsed-field gel electrophoresis, restriction pattern, nucleotide polymorphisms.

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Sammanfattning

Genetiska skillnader mellan *Listeria monocytogenes*-isolat

Bakterien *Listeria monocytogenes* kan orsaka sjukdomen listerios hos både människor och djur. Människor infekteras oftast genom att äta kontaminerade livsmedel. Bakterien är tålig mot kyla och kan tillväxa även i kylskåpstemperatur. Risklivsmedel är främst färdiglagade produkter som förvaras länge och sedan äts utan att värmebehandlas. Listerios kan manifesteras som meningoencefalit och sepsis. Infektion hos en gravid kvinna kan leda till att hon aborterar fostret eller att barnet föds sjukt eller insjuknar strax efter födelsen. Det är huvudsakligen människor med nedsatt immunförsvar som drabbas av listerios.

I detta avhandlingsarbete har olika metoder för att påvisa genetiska skillnader mellan *L. monocytogenes*-isolat använts.

L. monocytogenes kan under långa perioder leva i miljön i en livsmedelsindustri. Undersökning av genetiska skillnader mellan bakterieisolat som isolerats från ett mejeri under flera år med hjälp av pulsfältsgelelektrofores (PFGE) visade identiska klyvningsmönster. Detta tyder på att isolaten härstammar från en gemensam källa. PFGE-metoden användes också för att undersöka den genetiska variationen i ett litet material av *L. monocytogenes*-isolat från faeces från kliniskt friska mjölkkor.

Polymerase chain reaction och restriktionsenzymanalys (PCR-REA) av en del av en virulensgensregion i bakteriegenomet delade in serovar 1/2a-stammar i två grupper. Även sekvensering användes för karakterisering av *L. monocytogenes*-isolat. Både konventionell sekvensering av 1500 baspar av virulensgenen *inlB* och pyrosekvensering av två nukleotider i denna gen delade in *L. monocytogenes*-stammar i fyra grupper på samma sätt. Stammar tillhörande serovarerna 1/2a och 1/2c bildade en grupp, stammar tillhörande serovarerna 1/2b och 3b en annan grupp och serovar 4b-stammar delades upp i två grupper. Baserat på nukleotidskillnader i *inlB*-genen visade serovar 4b större diversitet än övriga undersökta serovarer.

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Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Unnerstad, H., Bannerman, E., Bille, J., Danielsson-Tham, M.-L., Waak, E. and Tham, W. 1996. Prolonged contamination of a dairy with *Listeria monocytogenes*. Netherlands Milk and Dairy Journal 50, 493-499.

II. Unnerstad, H., Romell, A., Ericsson, H., Danielsson-Tham, M.-L. and Tham, W. 2000. *Listeria monocytogenes* in faeces from clinically healthy dairy cows in Sweden. Acta Veterinaria Scandinavica 41, 167-171.

III. Unnerstad, H., Nilsson, I., Ericsson, H., Danielsson-Tham, M.-L., Bille, J., Bannerman, E. and Tham, W. 1999. Division of *Listeria monocytogenes* serovar 1/2a strains into two groups by PCR and restriction enzyme analysis. Applied and Environmental Microbiology 65, 2054-2056.

IV. Ericsson, H., Unnerstad, H., Mattsson, J. G., Danielsson-Tham, M.-L. and Tham, W. 2000. Molecular grouping of *Listeria monocytogenes* based on the sequence of the *inlB* gene. Journal of Medical Microbiology 49, 73-80.

V. Unnerstad, H., Ericsson, H., Alderborn, A., Tham, W., Danielsson-Tham, M.-L. and Mattsson, J. G. Pyrosequencing as a method for grouping of *Listeria monocytogenes* strains based on single nucleotide polymorphisms in the *inlB* gene. Submitted.

Abbreviations

A	adenine
AFLP	amplified fragment length polymorphism
ATP	adenosine triphosphate
bp	base pairs
C	cytosine
CAMP	Christie Atkins Munch-Petersen
CFU	colony forming units
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
ET	electrophoretic type
G	guanine
IDF	International Dairy Federation
ISO	International Organization for Standardization
MEE	multilocus enzyme electrophoresis
NMKL	Nordic Committee on Food Analysis
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PPi	pyrophosphate
RAPD	randomly amplified polymorphic DNA
REA	restriction enzyme analysis
RNA	ribonucleic acid
T	thymine
SNP	single nucleotide polymorphism

Introduction

Why spend several years doing research and writing a thesis about the bacterium *Listeria monocytogenes,* when the disease it is causing is quite unusual, both in humans and animals?

The reason is multifarious. Listeriosis may not commonly occur, but the disease is very serious, leading to death in about 30% of the human cases. Food is an important source of infection (Farber & Peterkin 1991) making *L. monocytogenes* interesting from a food hygienic point of view. The epidemiology of this organism is thrilling and investigations of it put hygienic aspects in focus. In the efforts to find the sources of infection to human cases and routes of contamination within the food processing industry, reliable methods for characterisation of the bacterium are useful. The wish to develop and apply such methods has been one prime mover. Pronounced curiosity, a preference for asking why, and a desire to find answers in the enormous field of microbiology are others.

General background

The disease

The bacterium *Listeria monocytogenes* causes the disease listeriosis in humans and animals. The disease does not commonly occur, but it is severe, and may lead to death. About 30 % of listeriosis patients die due to the infection (Hof et al. 1994). In Sweden, with almost 9 million inhabitants, about 30 cases of listeriosis per year are reported. However, the number of cases has increased during the year 2000 (Arneborn & Tham 2000) with 53 reported listeriosis patients (Malin Arneborn, pers. comm.). (Fig. 1).

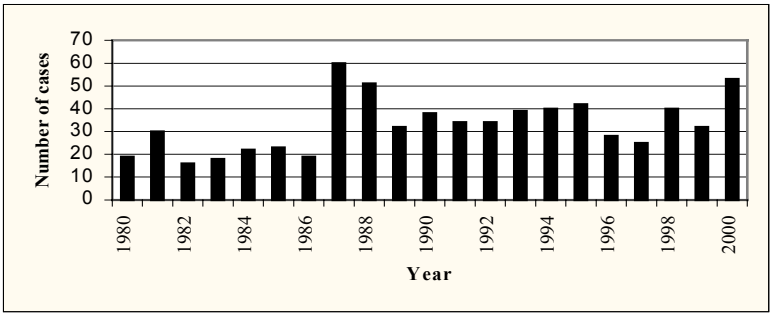

Figure 1. Reported cases of listeriosis.

Septicaemia and/or central nervous system infection are clinical manifestations that can be seen mainly in immunocompromised persons (Seeliger & Jones 1986). CNS involvement often includes meningitis or, in some cases, meningoencephalitis or abscesses in brainstem or spinal cord (Gellin & Broome 1989). Cancer, HIV-infection, diabetes, chronic alcoholism, treatment with immunosuppressive substances, old age and pregnancy are factors that may increase susceptibility to *L. monocytogenes* (Gellin & Broome 1989; Schuchat et al. 1991).

A pregnant woman may abort her foetus, or the child may be born seriously ill. The woman herself does not necessarily have to be ill, or she may show influenza-like symptoms (Gellin & Broome 1989). There are also reports of infection during pregnancy with isolates of *L. monocytogenes* from maternal blood cultures and influenza-like symptoms in the mother, but no evidence of infection in the foetus (McLauchlin 1990b). During pregnancy, the third trimester is the most frequent period for listeriosis infection (Gellin & Broome 1989), although it can occur throughout pregnancy (McLauchlin 1990b). When infants are infected in utero the symptoms occur within a few hours or a few days of birth (Gellin & Broome 1989). Neonatal listeriosis can also have a late onset with clinical manifestations several days or weeks after birth (Gellin & Broome 1989).

Although persons with underlying immunosuppressive conditions are most susceptible, there are reports of listeriosis in immunocompetent persons as well, (Gellin & Broome 1989; Aureli et al. 2000). Humans may harbour *L. monocytogenes* in the gastrointestinal tract and healthy carriers may shed *L. monocytogenes* for longer or shorter periods (Farber & Peterkin 1991).

A cutaneous form of listeriosis with papular lesions occurs in people working with infected animals, for instance veterinarians (Seeliger & Jones 1986).

Animals can be affected with encephalitis, septicaemia or abortion due to *L. monocytogenes* infection (Radostitis et al. 1994). In the encephalitis form of the disease, microabscesses can often be seen when the brainstem is examined histologically (Radostitis et al. 1994) (Fig. 2). The parenchymal lesions almost constantly spread to the meninges (Jubb et al. 1993). Multiple necroses in the liver (Fig. 3), spleen, endocardium and myocardium can be found in septicaemic animals and aborted foetuses (Radostitis et al. 1994). The disease is most often seen in domestic ruminants, particularly sheep, but several wild animals and non-ruminants are susceptible as well (Radostitis et al. 1994). In silage with increased pH value, *L. monocytogenes* may grow and be a source of infection for animals like sheep, goats and cattle (Radostitis et al. 1994).

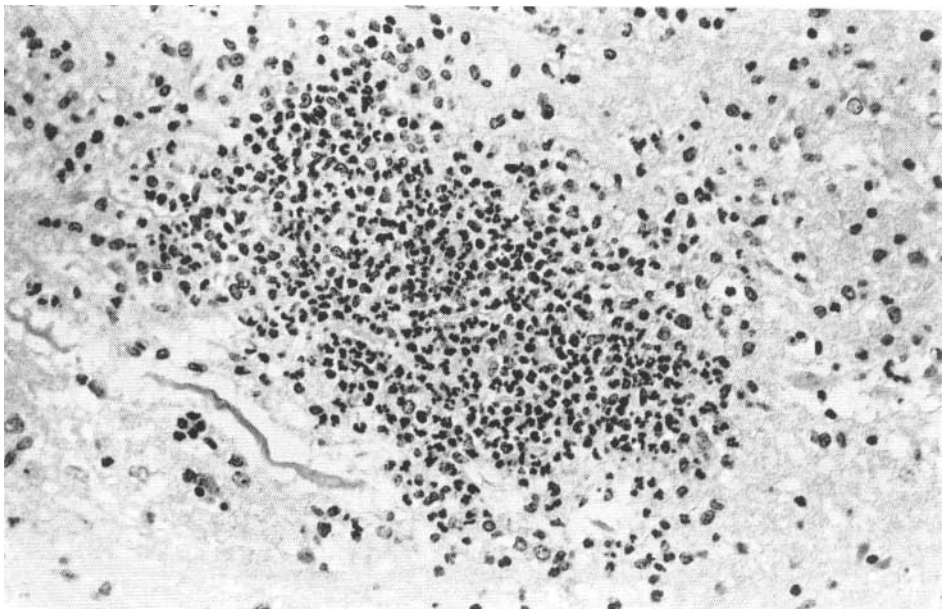

Figure 2. Microabscess in the brainstem of a sheep with listeriosis. Haematoxylin and eosin x 360. (Photo: Katarina Gustafsson)

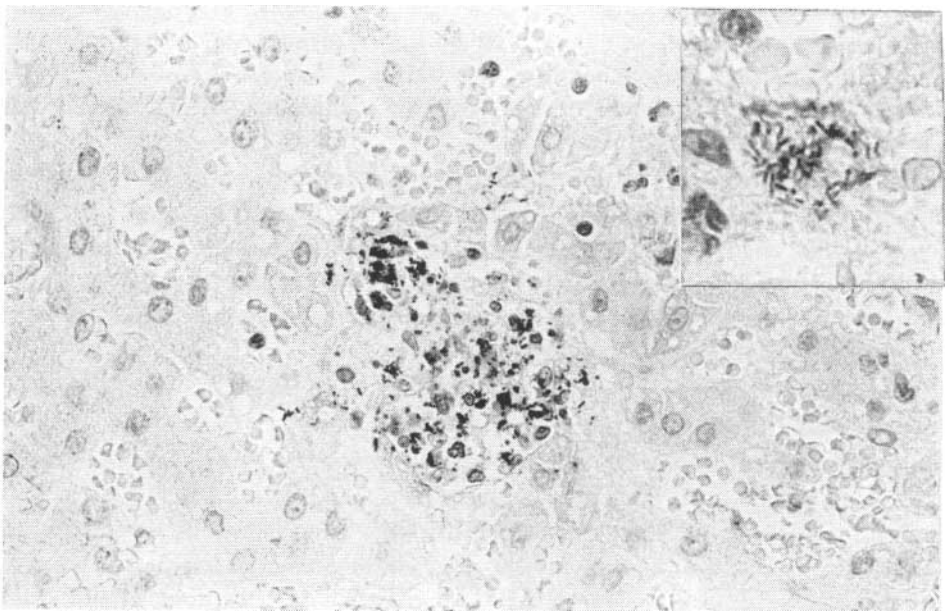

Figure 3. Focal hepatic necrosis in a marmoset with systemic listeriosis. Gram staining x 580. Inset: Intracellular, Gram positive rods of *L. monocytogenes* x 1440. (Photo: Katarina Gustafsson)

The bacterium

In the past

In March 1910, two rabbits were handed in for *post mortem* analysis at the section of bacteriology and pathological anatomy at the Veterinary Institute in Stockholm. The veterinarian Gustaf Hülphers performed the autopsies. In the liver he found a large number of grey-white necrotic foci sized as "poppy seed to pinheads" (Hülphers 1911). The spleen was a bit enlarged. From the liver he isolated a Gram positive bacterium which he called *bacillus hepatis*. He described the bacterium as a non spore forming, non acid fast short rod with growth temperature optimum at 37-38°C and with an ability to grow both aerobically and anaerobically. It was provided with a cilium and had a characteristic motility pattern. He tried a variety of growth media and performed inoculation studies on rabbits, mice, guinea pigs, hens, pigeon and horses, followed by autopsies. Since Hülphers' strain of *bacillus hepatis* has probably not been preserved for the future, we can not prove that what he isolated was *Listeria monocytogenes*, although it seems very likely.

With start in May 1924, an epidemic occurred among the laboratory rabbits at the Department of Pathology of the University of Cambridge (Murray et al. 1926). The rabbits lost weight and usually died suddenly. Autopsies were performed and a thorough investigation was done to clarify the cause of the disease. Liver necroses and monocytosis were some of the findings made by Murray and his co-workers. They concluded that a small Gram positive bacterium they had isolated was the cause of the disease. The bacterium was named *Bacterium monocytogenes*. The strain is preserved in the American Type Culture Collection, Rockville, Maryland, USA (ATCC 4428, ATCC 15313) and in the Catalogue of the National Collection of Type Cultures, London, UK (NCTC 10357).

In 1927, J. Harvey Pirie proposed the name *Listerella* as name for the genus. However, this name was already given to another organism. Since the term "Listerellosis" had become quite common in both human and veterinary pathology, he wanted a name as near his original suggestion as possible and in 1940 he proposed *Listeria* as a name for the genus and *Listeria monocytogenes* as the type species (Pirie 1940).

In the present

The genus *Listeria* comprises the species *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* (Seeliger & Jones 1986; Boerlin et al. 1992; Rocourt et al. 1992). The bacteria are regular, nonsporing, Gram positive rods, 0.4-0.5 µm in diameter and 0.5-2 µm in length with peritrichous flagella that makes them motile (Seeliger & Jones 1986). Colonies are 0.5-1.5 mm in diameter after 24-48 hours on nutrient agar. The bacteria are facultatively anaerobic and optimal growth temperature is between 30 and 37°C. Growth occurs at temperatures between 1°C and 45°C, at pH between 6 and 9 and in nutrient broth with up to 10% (w/v)

NaCl (Seeliger & Jones 1986). When cultured in nutrient broth at 20-25°C for 6-20 hours, a characteristic tumbling motility is shown where fast rotations in one place are followed by quick movements in different directions and again continued by rotations. Since the development of flagella is very weak at 37°C, motility studies are best done at room temperature (Seeliger & Jones 1986). Catalase is produced by all species and *L. monocytogenes*, *L. seeligeri* and *L. ivanovii* are haemolytic on blood agar. *L. monocytogenes* and *L. seeligeri* produce a narrow zone of haemolysis, while a wide zone or multiple zones of haemolysis surround colonies of *L. ivanovii*. *L. monocytogenes* ferments L-rhamnose but not D-xylose. *L. monocytogenes* is pathogenic to humans and animals and is therefore the most studied of the species of *Listeria*. *L. ivanovii* has been shown to cause disease in animals, especially in sheep (Seeliger & Jones 1986).

Table 1. Species of the genus *Listeria* (Seeliger & Jones 1986; Boerlin et al. 1992)

	L. monocy-togenes	*L. inno-cua*	*L. seeli-geri*	*L. welsh-imeri*	*L. gray*i	L. ivano-vii *subsp. ivanovii*	L. ivano-vii *subsp. londoni-ensis*
Gram positive rod	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	+
Tumbling motility	+	+	+	+	+	+	+
Haemolysis	+	-	+	-	-	+	+
L-rhamnose	+	d	-	d	-	-	-
D-xylose	-	-	+	+	-	+	+
CAMP-test (*Staph. aureus*)	+	-	+	-	-	-	-
CAMP-test (*Rhodococcus equi*)	-	-	-	-	-	+	+
Ribose						+	-
N-acetyl-ß-D-mannosamine						-	+

How to get infected

Humans are mainly infected by eating contaminated food (Farber & Peterkin 1991), making *L. monocytogenes* an interesting bacterium from a food hygienic point of view. *L. monocytogenes* not only survives, but can also grow at refrigerator temperatures. This quality makes cold-stored food with long shelf life, which will be eaten without further heat treatment, risk products. Even

small amounts of bacteria may multiply to large numbers during storage and if the product is eaten without cooking, there might be a risk of infection. *L. monocytogenes* has been found in uncooked ready-to-eat food products like soft cheese made of raw milk (Loncarevic et al. 1995) and vacuum packed cold smoked or gravad salmon/rainbow trout (Loncarevic et al. 1996b). Cole slaw (Schlech et al. 1983), soft cheese (Linnan et al. 1988; Bille 1990) and gravad and cold smoked rainbow trout (Ericsson et al. 1997) are examples of uncooked products that have caused outbreaks. Cooked ready-to-eat food products can be contaminated with *L. monocytogenes* after cooking and when eaten without further heat treatment there is a risk of infection. Paté (McLauchlin et al. 1991), pork tongue in jelly (Jacquet et al. 1995) and cooked medwurst (Loncarevic et al. 1997) are examples of heat-treated food products that have caused listeriosis in humans. Aureli et al. (2000) investigated an outbreak of febrile gastrointestinal illness among more than 1500 persons, mainly students from two primary schools. *L. monocytogenes*-contaminated corn and tuna salad served in the school cafeterias appeared to be the source of the outbreak.

Humans and animals may harbour *L. monocytogenes* in the intestinal flora without clinical symptoms (Husu 1990; Schönberg & Gerigk 1991). This is also shown in paper II, where faeces from clinically healthy dairy cows were investigated. Therefore, *L. monocytogenes* can be expected to be found wherever faecal contamination may occur.

Aspects on isolation of the bacterium

The ability of *L. monocytogenes* to survive and grow at low temperatures has been used for isolation purposes. Cold enrichment has been used for isolation from clinical samples (Gray & Killinger 1966). The sample is macerated in sterile water or nutrient broth and kept at 4°C. From the suspension, solid agar medium are plated repeatedly with several days interval and incubated at 37°C for 18-24 hours. The platings are continued for at least three months or until *Listeria* is isolated (Gray & Killinger 1966).

The time-consuming cold enrichment is nowadays replaced by faster isolation methods based on enrichment and selective media, where *Listeria* has the ability to grow but other bacteria are suppressed. Isolation of *L. monocytogenes* from material expected to be heavily contaminated with other bacteria, *e.g.* faecal samples and some food samples, is more difficult than isolation from normally sterile clinical specimens, like blood and cerebrospinal fluid. Samples known to contain a mixture of bacteria are often incubated in more than one step, starting with a liquid enrichment medium supplemented with antibiotics that are more suppressive to many other bacteria than to *Listeria*. In the next step, a certain volume of the enrichment medium is inoculated into a second enrichment broth that is incubated for another period. Finally, the enrichment broth is plated onto selective agar plates and incubated again.

Detection in food

The Nordic Committee on Food Analysis (NMKL) (NMKL 1999) has presented a method for enrichment, isolation and confirmation of *L. monocytogenes* in food products. A food sample of 25 g is homogenised in 225 ml enrichment broth I (LBI) containing nalidixic acid and acriflavine, and incubated at 30°C for 24 hours. Then 0.1 ml of LBI culture is transferred to 10 ml LBII enrichment broth (LBI with a higher acriflavine concentration) or Fraser broth. Incubation is done for 24 hours at 30°C if LBII is used and at 37°C if Fraser broth is used. One loopful of the enrichment culture is streaked on the surface of an Oxford agar plate and one loopful on a PALCAM agar plate. The plates are incubated at 37°C and examined after 24 and 48 hours. Typical colonies of *Listeria* spp. are small, black or grey and surrounded by a blackened zone. After 48 hours of incubation, the colonies show sunken centres. Five presumptive colonies from each plate are confirmed using investigation of Gram reaction, catalase production, tumbling motility, haemolysis of blood agar and fermentation of rhamnose and xylose.

Other methods for detection of *L. monocytogenes* in food are available, like the methods of IDF (International Dairy Federation standard 143A:1995) and ISO (International Organization for Standardization, ISO/DIS 11290-1, 1996).

Polymerase chain reaction (PCR) is a faster method for detection of *L. monocytogenes* in food than the methods based on enrichment and platings. However, food may contain substances like fat, protein and calcium ions in concentrations that inhibit the PCR reaction. Different procedures have been developed to reduce the inhibition problems and make the PCR detection suitable for use in food products like soft cheese, milk and gravad rainbow trout (Wernars et al. 1991; Rossen et al. 1992; Bickley et al. 1996; Ericsson & Stålhandske 1997). PCR detects DNA from *L. monocytogenes*, but the method does not result in isolation of a viable bacterial culture that can be further characterised.

Objectives

The objectives with the studies included in this thesis were to use genotyping methods to investigate routes of contamination for the bacterium *L. monocytogenes* and to study variations in a certain region of the genome. We wanted to investigate the possibilities to discriminate between different *L. monocytogenes* strains in a way similar to serotyping, but with methods based on nucleotide polymorphisms. Emphasis was put on revealing polymorphisms in the *inlB* gene and to use them for characterisation of strains within and between serovars.

Methodological considerations

In the present study, the methodological emphasis has been put on molecular biology based methods for typing of *L. monocytogenes*. Serotyping has been used throughout all five papers, as it is an established typing method. The outcome of the genotyping methods used was put in relation to the serotyping results and a similar grouping could often be done.

Isolation and confirmation of *Listeria*, paper II

In paper II *Listeria* was isolated from cow faeces. This was done mainly according to the procedure described by the International Dairy Federation (IDF 1990). This method is intended for analysis of dairy products. It might appear remote, but unpasteurised dairy products and cow faeces have some qualities in common, at least when it comes to the bacterial content. For example, in some goat cheeses made of raw milk coliform bacteria 44°C can be found in a number over 10^8 cfu/g (Tham et al. 1990) and in faecal samples from cows at least 10^5 cfu/g thermotolerant coliform bacteria have been isolated (Østensvik 1998). Total counts of aerobic bacteria can also be expected to be high both in raw milk cheeses and in faeces from cows. With the method used in paper II, 25 g faeces from each sample were analysed by enrichment in 225 ml of *Listeria* Enrichment Broth followed by spreading and incubation on the solid selective medium Oxford agar. Typical *Listeria* colonies on Oxford agar were subcultured on horse blood agar and tested for haemolysis, Gram reaction, cell shape, tumbling motility, fermentation of mannitol, rhamnose and xylose, production of catalase and hydrolysis of esculine.

Serotyping

Serotyping has long been an established phenotypic method for typing of *L. monocytogenes*. Seeliger and Höhne (1979) described the procedure based on agglutination of somatic (O) and flagellar (H) antigens of the bacterium with antisera from immunised rabbits. The species *L. monocytogenes* can be divided in 13 serovars - 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Seeliger & Jones 1986). The serogroups 1/2 and 3 are subdivided into serovars by the H-antigens and serogroup 4 is divided into serovars by the O-antigens (Schönberg et al. 1996).

The discrimination achieved by serotyping is often rather low, since most human isolates belong to either of the serovars 1/2a, 1/2b or 4b (Gellin & Broome 1989; Farber & Peterkin 1991; McLauchlin 1990a). The reproducibility of the serotyping method is better for serovars 1/2a and 4b than for the other serovars (Schönberg et al. 1996).

Only a few laboratories have a complete collection of antisera available, but serogroup 1/2 and 4 can be distinguished with Listeria O antiserum type 1 and type 4 (Difco Laboratories, Detroit, Michigan, USA). Complete serotyping is

still an advantage for laboratories working with characterisation of *L. monocytogenes* strains. Serotyping is important since the nomenclature is widely used and established. At the Department of Food Hygiene, we have a collection of *L. monocytogenes* strains. Many of the strains have kindly been serotyped by Prof. J. Bille and Dr. E. Bannerman, Centre National des Listeria, Switzerland. Serotyping is valuable for comparing the groups obtained by newer genotyping methods with serovars. Information about newly isolated strains can be correlated to the historical record and strain diversity over time can be investigated.

Serovar diversity

Serovar 4b strains have been dominating among isolates from human cases of listeriosis and strains of serovars 1/2a and 1/2b among isolates from food products. McLauchlin (1990a) reported domination of strains of serovar 4b among human listeriosis cases in the United Kingdom. During the period 1976-1985, 70% of human cases of listeriosis in Sweden were caused by strains of serovar 4b (Ericsson et al. 1996). Among strains isolated from food products, serogroup 1/2 has been reported to dominate (Farber & Peterkin 1991; Loncarevic et al. 1995; Loncarevic et al. 1996b). However, during recent years the distribution has changed, with strains of serogroup 1/2 increasing among human isolates (Gerner-Smidt et al. 1995; McLauchlin & Newton 1995; Loncarevic et al. 1998). In Sweden during 1986-1999, 45% of human isolates belonged to serovar 4b, 44% to serovar 1/2a and 10% to serovars 1/2b and 1/2c (unpublished results). The number of Swedish human isolates of serovar 4b seems to be decreasing markedly, since during 1998 and 1999 fewer than 30% and during 2000 only 10% belonged to serogroup 4 (unpublished results).

Pulsed-field gel electrophoresis, paper I, II and IV

Pulsed-field gel electrophoresis (PFGE) is a typing method with high discriminatory power. Whole genomic DNA is prepared in gel plugs. Then the DNA is subjected to enzymatic digest with a rare cutting restriction enzyme producing large fragments. The plugs are cast in an agarose gel and the DNA fragments are separated according to size in a pulsing electric field. In order to align with the electric field, the fragments start the procedure with orientation and deformation and this is followed by migration (Lognonne 1993). Since longer fragments need more time for orientation in the electric field than small fragments, the migration will be shorter for the large fragments (Lognonne 1993). Single field electrophoresis does not separate large fragments completely, since migration rate for large DNA molecules is independent of size in a uniform electric field (Lognonne 1993). After ethidium bromide staining, the result is presented as lanes with different banding patterns that vary depending on the number of restriction sites present in the genome. The whole genome is visualised in this way, meaning that if all the fragment sizes are added, the sum should be equal to the chromosome size of the bacterium analysed. The genome of *L. monocytogenes* is about 3 Mbp (Michel & Cossart 1992; Both et al. 1999).

In order to be as certain as possible that existing differences between strains really are found, more than one restriction enzyme can be used.

One problem that might arise is how to interpret the PFGE patterns. How much do the patterns have to vary in order to be called different? Tenover et al. (1995) have described how band differences could be interpreted and how the degree of relationship between isolates in a suspected outbreak could divide them in four groups. **Genetically indistinguishable** isolates have restriction patterns with the same number and sizes of the bands. **Closely related** isolates have band patterns that differ with a single genetic event – a point mutation creating or removing a restriction site, an insertion or a deletion of DNA. The band patterns of **possibly related** isolates differ with two independent genetic events. Isolates are considered **unrelated** if their PFGE patterns differ with three or more independent genetic events.

PCR and restriction enzyme analysis, paper III

The PCR and restriction enzyme analysis (PCR-REA) used in paper III is based on the production of a PCR product of 2916 bp, including part of the *inlA* gene and most of the *inlB* gene in the genome of *L. monocytogenes*. This PCR product was cleaved with the restriction enzyme *Alu*I. The fragments were separated in an agarose gel and visualised by ethidium bromide staining. The idea of restricting DNA, separating and visualising the fragments generated to obtain a fingerprint of the investigated bacterial strain is similar for PCR-REA and PFGE. However, with PFGE the whole genome is investigated whereas with PCR-REA only a short section of the genome is used to create the fingerprint. In paper III, exclusively serovar 1/2a strains were investigated with the PCR-REA method. We wanted to investigate whether serovar 1/2a could be divided into two groups, as had earlier been shown with serovar 4b strains (Ericsson et al. 1995). The expectation came true.

Sequencing, paper IV

Sequencing of the whole genome offers the most discriminatory information known today about bacteria. The discrimination might even be too good, if all nucleotide polymorphisms are considered. It is not, at least not yet, convenient to use sequencing of whole genomes for typing purposes, but sequencing of shorter regions or tags that can be used as markers is done easier. The diversity within the *inlA*-*inlB* region among strains of serovar 1/2a and 4b, respectively, visualised by the PCR-REA studies, was investigated more carefully in paper IV. Here, part of the PCR product in paper III was sequenced in strains representing serovars 1/2a, 1/2b, 1/2c, 3b and 4b. A segment of about 1500 bp comprising most of the *inlB* gene was sequenced in a total of 24 strains.

Pyrosequencing, paper V

Once again, the results of one study gave inspiration for the next. Within the 1500 bp segment sequenced in paper IV, a tag of a few bases was subjected to pyrosequencing in paper V. We discovered that the nucleotide polymorphisms in only two positions seemed to discriminate strains in basically the same groups as did sequencing of 1500 bp. The two variable positions are situated only two nucleotides apart and we presumed that pyrosequencing, a sequencing-by-synthesis method optimised for determination of short sequences, might be suitable.

In short, a sequencing primer is annealed to a single stranded DNA template and every nucleotide incorporation will be detected. The DNA template is incubated with four enzymes, DNA polymerase, ATP sulfurylase, firefly luciferase and apyrase. The four nucleotides, dATP, dTTP, dGTP and dCTP, are added one at a time in a certain order and every time a nucleotide is complementary to the DNA template it will be incorporated. In this reaction pyrophosphate (PPi) is released and converted to ATP by ATP sulfurylase. ATP is used for light production by help of luciferase and the amount of light produced is determined. Before a new cycle, starting with a new nucleotide addition, can proceed, the unincorporated nucleotides and the produced ATP must be degraded by the nucleotide-degrading enzyme apyrase. Every time a nucleotide is incorporated light is produced and since the order in which the nucleotides are added is known the template sequence can be determined. The result is presented as a Pyrogram™ where a light signal is shown as a peak.

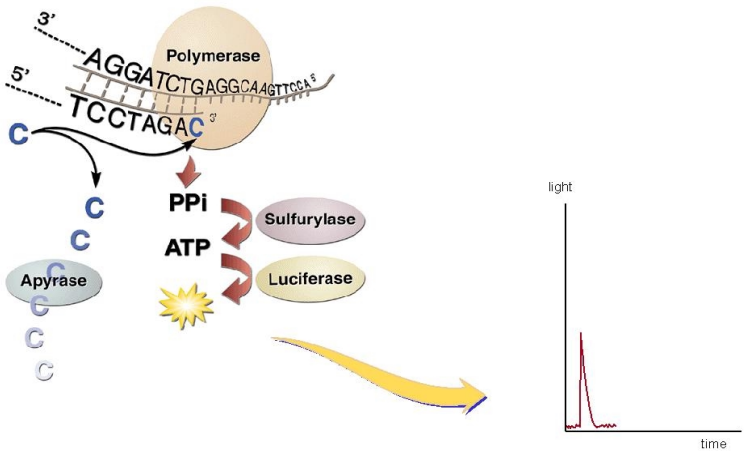

Figure 4. Schematic description of the pyrosequencing method.
(Illustration kindly provided by Pyrosequencing AB)

Absence of a peak indicates that the added nucleotide was not incorporated and a higher peak indicates that more than one nucleotide of the same kind was incorporated in succession. However, the sequencing procedure can not go on forever. Due to accumulation of intermediate products the activity of apyrase decreases, but at least 20 bases can be determined (Ronaghi et al. 1998).

The pyrosequencing method is used, for example, for determination of single nucleotide polymorphisms (SNPs) in the human genome (Alderborn et al. 2000). SNPs constitute the most common sequence variations in the human genome (Collins et al. 1998). At a SNP position there are different sequence alternatives, or alleles, with the least frequent allele appearing in a frequency of 1% or more (Brookes 1999; Alderborn et al. 2000). There are SNPs that can be correlated to increased risk of some diseases or to variations in drug responses. It is, however, probably the combined effect of several SNP alleles and environmental factors that together determines the risk of illness (Brookes 1999).

Results and discussion

Clonality

A bacterial clone can be described as a genetically homogeneous population of cells derived asexually from a single progenitor (Singleton & Sainsbury 1993). The most discriminative method for determining genetic similarities is sequencing of the genome, since it reveals differences as small as one nucleotide. However, total sequencing is not easily done, but fortunately, many different, more easily performed typing methods for investigations of genetic relationship between bacterial isolates are available. How much of the genome must be investigated in order to assign two isolates to the same clone? In the studies in this thesis, clone or clonal type is used if genetic differences can not be revealed after macrorestriction of total genomic DNA with three different enzymes and PFGE. Since all DNA is not stable over time and since most typing methods do not reveal all differences, we have to be satisfied by making it probable that two isolates are identical and derived from a common ancestor.

If more than one clonal type of *L. monocytogenes* is isolated from a food sample or from a listeriosis patient, investigations of the route of infection become more complicated. There are reports of the occurrence of more than one *L. monocytogenes* clonal type in the same food sample (Danielsson-Tham et al. 1993; Loncarevic et al. 1996a). From a listeriosis patient, different clonal types of *L. monocytogenes* were isolated from the blood and from the meninges (Tham et al. unpublished) and in paper II two clonal types were isolated from one faecal sample from a cow. Thus, it may be rewarding to type more than one colony from a primary culture medium, or to take more than one sample from a patient or food product.

Epidemiological applications for typing

Connections between food products and patients

One objective with typing of bacterial isolates is to reveal the degree of relationship among isolates and to use the results in epidemiological investigations. When investigating foodborne diseases the isolation of a bacterial species from patients and the isolation of the same species from a food product direct the suspicions towards that particular product. But a more specific identification is needed. If typing results of patient isolate and food isolate are identical, a link is established. A connection in time and space between the patient and the food product is, however, also wanted. If, *e.g.* cold smoked salmon or soft cheese is found in the refrigerator in a listeriosis patient's home in Sweden, these products are likely to be investigated. Further, if *L. monocytogenes* is found in an opened food package from the patient's home, examination of unopened packages of the same item from the local food shop, or relevant processing plant, is indispensable. Typing methods can help to identify the

probable route of contamination of food and infection of people as reliably as possible, but can we ever be 100% certain?

Outbreak detection and investigation

Small outbreaks can often be difficult to discover. Although listeriosis is mandatory to report in Sweden, recognition of an increased frequency of cases from a certain area requires alertness. At the Department of Food Hygiene many isolates from Swedish listeriosis patients are subjected to macrorestriction and PFGE. Detection of several isolates with identical restriction patterns will evoke the suspicion of a common source and an investigation of food products might be indicative. In combination with pattern conformity between patients' isolates and food isolates, a stronger epidemiological association is desirable. Knowledge of eating habits among patients and of distribution of incriminated food products is helpful.

By enhanced surveillance in diagnostic laboratories and hospitals in Wisconsin and some other states, human cases of listeriosis possibly associated with recalled dairy products were identified (Proctor et al. 1995). Four human isolates, two dairy product isolates and one isolate from the dairy environment displayed identical restriction patterns after digestion with *Asc*I and PFGE, indicating that the patients were part of an outbreak (Proctor et al. 1995).

Surveillance of human listeriosis can be useful to detect outbreaks in an early stage and can, for instance, be done by the phenotypic typing methods serotyping and phage typing (Jacquet et al. 1995). A major outbreak of listeriosis was observed in France in 1992, when an increased number of cases was reported to be caused by serovar 4b and a special phagovar (Jacquet et al. 1995). Of 279 human strains 247 displayed identical combination of patterns after macrorestriction with three enzymes and PFGE. Strains with the same patterns were also obtained from 154 food samples, with pork tongue in jelly contributing with a major part of the strains (Jacquet et al. 1995).

In outbreak investigations a fast method, like serotyping, can be useful as a first-step typing method for screening strains from many people and from many probable sources. Isolates from food items suspected to be the source of infection need, however, to be typed with a more discriminating method for identification of the epidemic strain. Several outbreaks of listeriosis have been associated with strains of serovar 4b (Schlech et al. 1983; Linnan et al. 1988; Bille 1990; Jacquet et al. 1995). Many outbreak isolates have been subjected to different typing methods in order to confirm and investigate their relationship (Boerlin & Piffaretti 1991; Nocera et al. 1993; Boerlin et al. 1996). All of 34 epidemiologically related strains of serovar 4b, associated with a Swiss outbreak 1983-1987, were clustered in a unique group by each of the three typing methods ribotyping, restriction enzyme analysis of chromosomal DNA and multilocus enzyme electrophoresis (MEE) (Nocera et al. 1993).

In a large outbreak of febrile and gastrointestinal listeriosis with more than 1500 persons showing symptoms, consumption of a corn and tuna salad was

epidemiologically associated with illness. Strains from humans, food and environment were indistinguishable using macrorestriction and PFGE, and randomly amplified polymorphic DNA, supporting the epidemiological link even more (Aureli et al. 2000).

Investigation of contamination routes

Typing methods can also be used when investigating routes of contamination in certain environments. The spread of foodborne pathogens, like *L. monocytogenes*, in food processing environments is interesting to clarify in order to show if a wide variety of clonal types are just passing through the plant, or if the contamination flora is firmly established. In the case of a house flora, consisting of a specific clonal type, the eradication regime may be quite different than in a situation where new strains keep coming in to the plant continuously. In paper I, the PFGE method has been used for typing purposes in order to reveal the diversity among *L. monocytogenes* strains isolated from a dairy plant (see below).

A small number of isolates from faeces from clinically healthy cows were investigated with PFGE (paper II). Three cows living at the same farm harboured the same clonal type of *L. monocytogenes,* indicating a common source in the environment. Interestingly, one of them also harboured a second clonal type.

L. monocytogenes in food processing environments

The ability of *L. monocytogenes* to survive, and sometimes grow, under suboptimal conditions makes it a survivor in food processing environments. Once established, massive efforts are needed in order to eradicate the pathogen from a food plant. Paper I deals with contamination problems in a dairy. The question at issue in that investigation was whether isolates from cheeses, wash water and dairy environment were the result of a continuous recontamination from outside, or if the dairy, during at least seven years, was harbouring a domestic flora of genetically identical bacteria with a common origin. Since the investigated isolates belonged to the rather unusually occurring serovar 3b, we expected them to have a common origin. Further investigations of their genetic similarities by PFGE were performed. All 10 dairy strains collected between 1988 and 1995 showed identical restriction patterns after enzymatic digests with the three endonucleases *Apa*I, *Sma*I and *Asc*I, respectively, supporting the theory of a domestic clonal type. Although the dairy made intensive efforts to eliminate the source of contamination, the clonal type remained in the dairy plant for at least seven years, although a reduction in the number of positive samples was seen.

There are several reports of long survival of the same clonal types of *L. monocytogenes* in food processing plants. During one year, nine cases of listeriosis were detected in the province of Värmland in Sweden. *L. monocytogenes* isolates from the patients and from gravad and cold smoked rainbow trout were characterised with PFGE. The same clonal type of *L. monocytogenes* isolated from six of the patients was also isolated from gravad

rainbow trout in one of the patient's refrigerator, from unopened packages of gravad rainbow trout of the same brand and from the packing machine at the producer of the processed rainbow trout (Ericsson et al. 1997). The products of this fish processing plant may have been contaminated in the packing machine. It is not unlikely that the plant harboured the same clonal type of *L. monocytogenes* at least during the one year the outbreak continued.

A Norwegian investigation of *L. monocytogenes* isolates from sea water, fish slaughterhouse environment, smokehouse environment, fish before and during processing in the smokehouse and vacuum-packed, smoked salmon by multilocus enzyme electrophoresis revealed 11 electrophoretic types (ET) of *L. monocytogenes* (Rørvik et al. 1995). One ET of *L. monocytogenes* was found in environmental samples and fish samples from the smokehouse during the whole investigation period of eight months, indicating that a reservoir of *L. monocytogenes* had been established in the smokehouse. The same ET was found in vacuum-packed smoked salmon. Other ETs were found in isolates from seawater, the slaughterhouse, and fish coming in to the smokehouse.

Autio et al. (1999) characterised isolates of *L. monocytogenes* from fish and environment in a cold-smoked rainbow trout processing plant by PFGE. Brining and postbrining areas were found to be the most contaminated sites in the plant and the fish seemed to be contaminated during processing. Some clonal types were predominating in the plant environment and could be detected both before and during daily production.

Investigations of *L. monocytogenes* isolates from the meat processing industry by multilocus enzyme electrophoresis showed that different ETs often were found in animals and meat products, indicating that the meat products were contaminated by environmental strains during the production process rather than by animal strains (Boerlin & Piffaretti 1991).

Nesbakken et al. (1996) used multilocus enzyme electrophoresis to characterise isolates from deboned fresh meat, cold cuts and production environment from meat processing plants. They found two clone variants that seemed to have colonised one plant for at least four years.

Closely related strains of *L. monocytogenes* were present in the environment of two French pork slaughtering and cutting plants during a period of one year, in spite of cleaning and disinfection (Giovannacci et al. 1999).

Perhaps some strains of *L. monocytogenes* are more prone to be established in a certain environment than others. For some reasons some strains seem adapted for a life in the niche of a certain food processing plant.

The ability of *L. monocytogenes* to form biofilm is one quality that facilitates the establishment of the bacterium in food processing plants. By forming biofilm *L. monocytogenes* can attach to different types of surfaces used in food processing environments and the biofilm formation makes the sanitation procedure harder (Ronner & Wong 1993; Blackman & Frank 1996). Adherence can occur even in refrigeration temperature (4°C) and a contact time of less than one hour can be enough for attachment (Mafu et al. 1990).

Major clusters of *L. monocytogenes* strains visualised by different methods

Many genotyping methods have been used on *L. monocytogenes*. Most of them are more discriminative than serotyping and, thus, they are more useful for investigations of outbreaks and contamination routes. Besides the discriminatory ability, many genotyping methods, as exemplified below, create two general clusters of strains.

The genotyping methods investigated in papers III, IV and V have in common the use of the sequence polymorphisms in the region of the genes *inlA* and *inlB* for discriminating strains of *L. monocytogenes*. According to papers IV and V, nucleotide polymorphisms in the *inlB* gene can be used for dividing strains of *L. monocytogenes* in four groups. The groups were correlated to serovars. Strains of serovars 1/2a and 1/2c formed one genomic cluster, strains of serovars 1/2b and 3b another, while serovar 4b strains were divided into two groups. Principally the same divisions have been made using other typing methods, with exception for the two serovar 4b groups. According to the phylogenetic analysis in paper IV also strains of serovar 1/2a could be divided into two groups, in agreement with the results in paper III, although not as distinctly as the 4b-strains.

Brosch et al. (1994) have shown that division of *L. monocytogenes* strains by macrorestriction and PFGE can be correlated to flagellar antigen types and, consequently, also to serovars. They obtained two genomic divisions with strains of serovars 1/2a, 1/2c, 3a and 3c in one, and strains of serovars 1/2b, 3b, 4b, 4d and 4e in the other.

PCR-amplification is the basis for some typing methods. Vines et al. (1992) have used PCR-amplification and restriction fragment length polymorphisms in four virulence associated genes, *hlyA*, *iap*, *mpl* and *prfA*, to categorise *L. monocytogenes* strains into two groups with strains of serovars 1/2a, 1/2c and 3a in one group, and 1/2b, 3b and 4b in another. This method utilises a window of the genome for typing. In randomly amplified polymorphic DNA (RAPD), however, the whole genome is used and short primers with a randomly chosen sequence are annealed under low stringency conditions. When primers anneal 200-2000 bp apart on opposite strands amplification may occur (Farber 1996). An advantage with RAPD is that knowledge of the target DNA sequence is not needed (Farber 1996). For good reproducibility the conditions must be carefully controlled and standardisation problems must be overcome (Boerlin et al. 1995; Farber 1996). The RAPD method has been applied on *L. monocytogenes* strains in several studies (MacGowan et al. 1993; Farber & Addison 1994; Boerlin et al. 1995; Wernars et al. 1996). According to the investigation of Boerlin et al. (1995) the RAPD types were not completely correlated to specific serovars. However, with primer PB1 the presence of a band of about 2300 bp was demonstrated in isolates of serovars 1/2a, 1/2c, 3a and 3c that was missing in isolates of serovars 1/2b, 3b, 4a, 4b, 4c, 4d, 4e and 7.

In ribotyping, probes are used for recognition of ribosomal genes (Farber 1996). Isolated bacterial DNA is enzymatically digested and the fragments are

separated by gel electrophoresis. The DNA is then transferred to a membrane and hybridisation is performed with labelled ribosomal RNA (rRNA) or rDNA probes. Since the genes coding for rRNA are highly conserved, these genes are very similar between different bacterial species and probes based on the sequence of *E. coli* 23S, 16S and 5S can be used for typing of different species. Using restriction with the enzyme *Eco*RI and hybridisation with a labelled rRNA operon from *E. coli* Bruce et al. (1995) tested 1346 *L. monocytogenes* strains and obtained 50 ribotype patterns. Wiedmann et al. (1997) observed 23 different ribotypes among 133 strains subjected to ribotyping. Ribotyping in combination with investigation of restriction fragment length polymorphisms in the three virulence genes *hly*, *actA* and *inlA,* resulted in separation of the *L. monocytogenes* strains into three distinct lineages (Wiedmann et al. 1997). Ribotyping of 80 strains produced two genomic clusters with strains of serovars 1/2a and 1/2c in one, and strains of serovars 1/2b, 3b, 4b and 4d in the other (Swaminathan et al. 1996).

In amplified fragment length polymorphism (AFLP) total DNA is digested with a restriction enzyme and an oligonucleotide adapter is ligated to the restriction sites (Ripabelli et al. 2000). PCR amplification is performed with primers corresponding to the base sequence in the adapter, restriction site and one or two nucleotides in the original DNA. The amplified fragments are separated with gel electrophoresis. With this method, Ripabelli et al. (2000) could divide *L. monocytogenes* strains into two genetic groups with strains of serovars 1/2a and 1/2c in one group and serovars 1/2b, 3b and 4b in another.

In multilocus enzyme electrophoresis (MEE) the electrophoretic mobility of different cellular enzymes is used for characterisation of isolates (Selander et al. 1986). MEE detects differences in the genetic level in an indirect way. Piffaretti et al. (1989) showed clustering of strains of serovars 1/2b, 4b and 4a in one division and 1/2a and 1/2c in another using MEE.

Strains of serovars 1/2b and 3b and serogroup 4 have the same flagellar antigens (A, B and C), whereas strains of serovar 1/2a and 3a have flagellar antigens A and B and strains of serovars 1/2c and 3c flagellar antigens B and D (Seeliger & Höhne 1979). As is exemplified above, grouping according to flagellar antigens correlate in a distinct way with grouping based on polymorphisms in a variety of genes, since the division of serovars in two major groups, at least, is principally the same using different methods. The genetic development seems to be congruent with serovar diversity.

The *inlA-inlB* region

L. monocytogenes is an intracellular pathogen that is able to induce its own uptake into normally non-phagocytic mammalian cells. The ability to be internalised by both phagocytes and non-phagocytic cells is important for the development of systemic infection caused by the bacterium (Braun et al. 1998). The genes *inlA* and *inlB*, located next to each other, encode two proteins that are

involved in internalisation. The similarities between these genes indicate that they originate from a common ancestral sequence (Gaillard et al. 1991). Gaillard et al. (1991) who first described the region, termed it *inl* for internalisation.

The gene *inlA* consists of 2232 bp and encodes a protein of 744 amino acids. The protein, originally named internalin or inlA, mediates the entry of *L. monocytogenes* into epithelial cells (Gaillard et al. 1991). Internalin is a surface protein and contains two regions of repeats, region A and region B (Gaillard et al. 1991).

The 1890 bp gene *inlB* is situated downstream of *inlA* and encodes a 630 amino acids protein. The inlB protein mediates invasion in, for instance, cultured hepatocytes and some epithelial and endothelial cell lines (Dramsi et al. 1995; Ireton et al. 1996; Braun et al. 1998; Greiffenberg et al. 1998). Inert particles and non-invasive bacteria coated with inlB may also be capable of entering mammalian cells (Braun et al. 1998). Apart from being a surface protein, inlB is also present in culture supernatants, but the ability to promote invasion by the released form of inlB is limited (Braun et al. 1998).

Investigations of polymorphisms in the *inlA-inlB* region

Sequence determinations in papers IV and V were concentrated on the *inlB* gene, whereas PCR-REA in paper III included part of the *inlA* gene as well. A PCR amplicon of 955 bp of the downstream end of *inlA*, 85 bp of the space between *inlA* and *inlB*, and 1876 bp of *inlB*, in total 2916 bp, was enzymatically digested with *Alu*I and the fragments were separated by gel electrophoresis. Applied on serovar 4b strains this PCR-REA method had earlier resulted in two restriction fragment patterns (Ericsson et al. 1995). We wanted to test this grouping method also on serovar 1/2a strains and the result was a clear division in two groups. Since strains of serovars 4b, 1/2a and 1/2b are more commonly isolated from human cases than strains of other serovars (Gellin & Broome 1989; Farber & Peterkin 1991), subdivision of serovars 4b and 1/2a could be useful for discrimination of strains. Studies of the nucleotide sequences of most of the *inlB* gene of strains of different serovars in paper IV confirmed the grouping of both serovar 4b and 1/2a strains, while nucleotide sequence polymorphisms within the other serovars were less distinct or not present at all. Poyart et al. (1996) used a 760 bp sequence in the downstream end of *inlA* for enzymatic digest with *Acc*I, *Alu*I and *Rsa*I and no differences could be detected between the obtained restriction patterns in the 68 analysed strains of *L. monocytogenes*. This low degree of polymorphism in the downstream end of *inlA* indicated that the polymorphisms observed in our PCR-REA studies probably are situated within the *inlB* gene. This was one reason for us to concentrate the nucleotide sequencing investigation to the *inlB* gene. The two serovar 4b groups were established in the PCR-REA study and confirmed both by sequencing and also with the pyrosequencing method used in paper V. Pyrosequencing could not distinguish between strains of serovars 1/2a and 1/2c or between 1/2b and 3b. This result was expected since the positions used for pyrosequencing did not vary

within these groups according to the sequencing results in paper IV. Pyrosequencing of 4b strains, however, revealed interesting findings. In the investigated region the pyrograms showed adjacent peaks with half the expected heights indicating a mixture of two nucleotides in some positions. A combination of C and T in position 1575 and of G and T in position 1578 of the *inlB* gene appeared unique for strains of the 4b-I group, whereas T in position 1575 and a combination of G and T in position 1578 determined strains of the 4b-II group. In conclusion, pyrosequencing of only two nucleotides in the *inlB* gene of serovar 4b strains yielded, almost exactly, the same two groups as sequencing of 1500 bp of *inlB* and PCR-REA of 2916 bp in the *inlA*-*inlB* region.

Serovar 4b diversity

Sequencing of most of the *inlB* gene in paper IV showed that there was a higher degree of diversity among strains within serovar 4b than among strains within the other serovars. This was shown both for the nucleotide sequence and at the amino acid level. Amino acid differences were seen only within serovars 4b and 1/2a, with the largest diversity within serovar 4b.

With the pyrosequencing investigation (paper V), we wanted to test if grouping of strains based on two positions in the *inlB* gene, was in agreement with grouping based on sequencing of *inlB* and, for serovar 4b strains, also based on PCR-REA. The finding of nucleotide mixtures in some positions was an unexpected discovery that led us into new investigations. In order to test if the nucleotide mixtures in positions 1575 and 1578 were true or just indications of errors in the method, we sequenced a 104 bp segment from a number of individual clones from one 4b-I and one 4b-II strain. The cloning and sequencing also resulted in more than one nucleotide sequence. Finally, Southern blot experiments were performed where an *inlB* DNA probe hybridised twice to the enzymatically digested DNA from the serovar 4b strains, but only once to digested DNA from strains of serovars 1/2a, 1/2b, 1/2c and 3b. After our experiments were concluded, a sequence of the *ami* gene from a serovar 4b strain was deposited in GeneBank containing a region similar to the 3'-end of *inlB*. Taken together, these results indicate that a segment similar to the investigated part of *inlB* is present in the genomes of serovar 4b strains, but no such indications were found for strains of the other serovars. We presume that the sequence causing the nucleotide alternatives and the second hybridisation band in serovar 4b strains can be found within the *ami* gene.

Concluding remarks

L. monocytogenes can remain established in food processing plant environments for several years. Investigations of genetic diversity among isolates of the bacterium facilitate the visualisation of bacterial spread within the environment.

The gene *inlB* seems to be well suited for typing of *L. monocytogenes*. Sequence analyses of *inlB* and serotyping gave congruent results when used for grouping of strains of *L. monocytogenes*. Since the polymorphisms in two positions of the gene reflected division based on polymorphisms in most of the gene, nucleotide determination of two positions was sufficient to divide *L. monocytogenes* strains in a way reflecting major serovar clusters.

Serovar 4b strains were differentiated from other strains based on nucleotide polymorphisms in the *inlB* gene and the heterogeneity in this gene within the serovar was used for division in two groups. Pyrosequencing analyses and hybridisation experiments indicated the presence of a sequence similar to *inlB* elsewhere in the genome of serovar 4b strains that could not be found among strains of the other investigated serovars.

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