USE OF MOLECULAR GENETICS TO STUDY THE DETECTION AND PATHOGENICITY OF FOODBORNE *LISTERIA MONOCYTOGENES*

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DEDICATION

To my family and R.C.M.

ABSTRACT

Cryptic plasmids ranging from 2.0 to 100 kb in size were isolated from 25 out of 122 Listeria monocytogenes strains, and from 7 out of 11 strains of other Listeria species.

Of 2500 clones of a genomic library of L. monocytogenes 81-861 generated in Escherichia coli cells, 5 clones were identified in which ß-hemolytic activity was stably expressed. Testing by intraperitoneal injection showed that these clones were lethal to mice. Restriction mapping of the inserts of the recombinant plasmids showed that, apart from a 650-bp internal Hind III fragment in 2 inserts, there were no other common sites. No homology was demonstrated between the DNAs of the inserts when Southern blots of restriction digests of the 5 plasmids were probed, though homology was demonstrated between the L. monocytogenes listeriolysin O gene and the DNA of one insert. The evidence suggests that at least one additional ß-hemolysin, other than listeriolysin O, exists in this strain of L. monocytogenes, and that it may be a virulence factor.

Using a direct colony hybridization procedure on hydrophobic grid-membrane filters (HGMFs), the inserts of the recombinant plasmids were screened, and a DNA probe specific for L. monocytogenes was identified. After labelling with horseradish peroxidase and colour development of the chromogen, a commercial counter (HGMF Interpreter) was able to detect and count the organism electronically. When the efficacy of the chromogen-labelled DNA probe method on HGMFs was compared with the conventional method for three artificially-inoculated foods, there were no significant differences ($\alpha = 0.05$) shown in the recovery of L. monocytogenes from the foods.

RESUMÉ

Parmi 122 souches de Listeria monocytogenes et 11 souches d'autres espèces de Listeria, 25 et 7 ont respectivement permis l'isolement de plasmides dissimulés de l'ordre de 2,0 à 100 kb.

Des 2 500 clones d'une librairie de gènes de L. monocytogenes 81-861 produit dans des cellules de Escherichia coli, 5 ont été reconnus comme ayant une activité bêta-hémolytique stable. Des essais pratiqués par injection péritonéale ont révélé que ces clones étaient létals chez la souris. La cartographie de restriction des insérats des plasmides recombinés a démontré l'absence de tout autre site commun, exception faite d'un fragment interne de 650 bp coupé par l'enzyme Hind III dans deux insérats. Aucune homologie entre les ADN des insérats n'a pu être décelée par sondage génétique des Southern blots pratiqués sur les digestas de restriction des 5 plasmides, bien qu'une homologie ait été établie entre le gène de la listériolysine O de L. monocytogenes et l'ADN d'un insérat. Ces résultats donnent à penser que, mis à part la listériolysine O, il existe au moins une autre hémolysine β dans cette souche de L. monocytogenes qui pourrait être un facteur de virulence.

Les insérats des plasmides recombinés ont été examinés par hybridation directe des colonies sur membrane filtrante avec grille hydrophobe (HGMF) et une sonde d'ADN spécifique de L. monocytogenes a été identifiée. Suite au marquage par la peroxydase de raifort et au développement de la couleur de l'enzyme, un compteur commercial (le HGMF Interpreter) a pu déceler et dénombrer le microorganisme électroniquement. La comparaison de l'efficacité de la méthode sur HGMF par sonde d'ADN marqué par chromogène et de la méthode traditionnelle pour trois aliments inoculés artificiellement, n'a révélé aucun écart notable (α = 0.05) dans la récupération de L. monocytogenes dans des aliments.

CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. Twenty percent of L. monocytogenes strains contain cryptic plasmids with molecular sizes ranging from 2.0 to 100 kb. Sixty-four percent of other Listeria species contain plasmids ranging from 58 to 71 kb in size.

2. A determinant for β-hemolytic activity from L. monocytogenes 81-861 genomic DNA may be a sufficient cause of virulence when expressed in a non-pathogenic bacterium, E. coli.

3. There is genetic evidence for the presence of one or more β-hemolysins, other than listeriolysin O, in L. monocytogenes 81-861.

4. A DNA probe specific for L. monocytogenes was isolated and identified.

5. Procedures for performing direct colony hybridization on HGMFs were developed for use with either radioisotope- or chromogen-labelled DNA probes.

6. The electronic detection and enumeration of microorganisms by means of a chromogen-labelled DNA probe was demonstrated using a commercial counter (HGMF Interpreter).

7. The procedure using the chromogen-labelled DNA probe is applicable to the detection of L. monocytogenes in inoculated foods.

8. The HGMF is also useful for the determination of probability of purity of bacterial cultures, and for the screening and storage of recombinant clones.

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AP	alkaline phosphatase
AOAC	Association of Official Analytical Chemists
BCIP	5-bromo-4-chloro-3-indolylphosphate
BHI	brain heart infusion
BMC	Boehringer Mannheim Canada
BSA	bovine serum albumin
CAMP	Christie, Atkins and Munch-Petersen
CAP	calf intestinal alkaline phosphatase
CHO	Chinese hamster ovary
D	decimal reduction value
Da	Daltons
Dig	digoxigenin
DMSO	dimethylsulfoxide
DNP	dinitrophenyl
DTT	dithiothreitol
EDTA	ethylenediaminotetraacetic acid
EIA	enzyme immunosorbent assay
ELA	enzyme-linked antibody
ELISA	enzyme-labelled immunosorbent assay
EMB	eosin methylene blue
EtBr	ethidium bromide
ETEC	enterotoxigenic E. coli
FCM	flow cytometry
HGMF	hydrophobic grid-membrane filter
hly	the hemolysin gene
Hly	hemolysin
HPB	Health Protection Branch
HRP	horseradish peroxidase
HTST	high temperature, short time pasteurization
IPTG	isopropyl-ß-D-thiogalactopyranoside
LD_{50}	lethal dose for 50% of the test animals
LEB	Listeria enrichment broth
LM	low melting
LPM	lithium chloride-phenylethanol-moxalactam
LT	heat-labile enterotoxin
LTB	Listeria test broth
MAB	monoclonal antibody
MF	membrane filter
MMA	modified McBride's agar
MPA	monocyte-producing agent
MPN	most probable number
MPNGU	most probable number of growth units
MVJ	modified Vogel-Johnson's medium
MW	molecular size
NBT	nitroblue thiazolium
NCM	nitrocellulose membrane
ORF	open reading frame
PBS	phosphate buffered saline

PCA	plate count agar
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethyleneimine
PMNL	polymorphonuclear leukocyte
PVP	polyvinylpyrrolidone
RB-high	restriction buffer, high ionic strength
SDS	sodium dodecyl sulfate
SEB	staphylococcal enterotoxin B
SLO	streptolysin O
SOD	superoxide dismutase
SSC	standard saline citrate
ST	heat-stable enterotoxin
TA	tryptose agar
TAE	tris-acetate electrophoresis buffer
TE8	Tris-EDTA (pH 8.0)
TELT	Tris-EDTA-LiCl-Triton X-100 buffer
TFB	transformation buffer
Tn	transposon
TPB	tryptose phosphate broth
TSA	tryptic soy agar
TSB	tryptic soy broth
TSBYE	tryptic soy broth with yeast extract
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

1. INTRODUCTION

1.1 Listeria monocytogenes

1.1.1 The Organism

Listeria monocytogenes is a Gram-positive, nonsporing, facultatively anaerobic rod first isolated from infected laboratory rabbits and guinea-pigs by Murray et al. (1926). It grows in a temperature range of 0.5 to 50°C (Junttila et al., 1988), and a pH range of 4.7 to 9.2 (Petran and Zottola, 1989), with optimum growth occurring between 30 and 37°C at pH 7.0 (Petran and Zottola, 1989). In the latest edition of Bergey's Manual of Determinative Bacteriology, it is placed with Lactobacillus, Erysipelothrix, Brochothrix and other genera, in a section entitled "Regular, Nonsporing Gram-positive Rods" (Seeliger and Jones, 1986). L. monocytogenes is catalase-positive and oxidase-negative, and produces a ß-hemolysis on blood agar. The hemolysin acts synergistically with the ß-hemolysin of Staphylococcus aureus on sheep erythrocytes; the substance mediating this effect is known as the CAMP factor, named after Christie, Atkins and Munch-Petersen (1944) who first described the phenomenon in group B streptococci. The organism has peritrichous flagella which give it a tumbling motility which occurs only when the organism is grown between 20 and 25°C. Under these circumstances, flagellin is produced and assembled at the cell surface; flagellin production is markedly reduced at 37°C (Peel et al., 1988). The colonies demonstrate a characteristic blue-green sheen by obliquely transmitted light (Henry, 1933). L. monocytogenes is widely present in plant, soil and surface water samples (Weis and Seeliger, 1975), and has also been found in silage, sewage, slaughter-house waste, milk of normal and mastitic cows, and human and animal feces. L. monocytogenes isolates from animals have been reported in cattle, sheep, goats and poultry as well as infrequently from wild animals (Gray and Killinger, 1966).

Both the intra- and intergeneric taxonomy of bacteria of the genus Listeria have been problematical for a number of years. Reviews on the topic include those by Jones (1975, 1988),

Seeliger (1984), and McLauchlin (1987). As concluded from the numerical taxonomic studies, as well as the more recent DNA homology and 16S rRNA cataloguing results, the present taxonomic position of the genus Listeria is as follows (Jones 1988; Rocourt et al., 1987; Rocourt, 1988):

1. It includes the species L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, L. grayi and L. murrayi.

2. L. denitrificans is excluded from the genus and transferred to a new genus, Jonesia, as J. denitrificans.

3. The genus is closely related to the genus Brochothrix. Both of these genera occupy a position between Lactobacillus and Bacillus, and are more distantly related to Streptococcus, Lactococcus, Enterococcus, Staphylococcus, Kurthia, Gemella, and Erysipelothrix.

In the 1950s, Seeliger (1958) and Donker-Voet (1959) extended the work of Paterson (1940) to devise the present serotyping system for the genus Listeria (Table 1-1).

Plasmids are found relatively infrequently in strains of Listeria spp., perhaps due to the use in primary isolation media of plasmid-curing agents such as acridine. Of 32 Listeria strains (29 L. monocytogenes, 2 L. grayi, 1 L. murrayi) examined, 7 (4 L. monocytogenes, and the 3 other strains) contained a cryptic plasmid of 38.5 Md (Perez-Diaz et al., 1982). Restriction enzyme analyses suggested that these plasmids formed a single molecular species. No phenotypic character was related to the presence of a plasmid.

1.1.2 Relation to Foodborne Disease

L. monocytogenes causes a disease of humans and animals named listeriosis. Human listeriosis is characterized by stillbirth or neonatal death in perinatal cases (Seeliger, 1987, 1988), and by cutaneous lesions, septicemia, meningitis, and encephalitis in adults, particularly in the

Table 1-1. Serovars of the genus Listeria

Paterson[a]	Seeliger[b] Donker-Voet[c]	O Antigens											H Antigens				
1	1/2a	I	II	(III)									A	B			
	1/2b	I	II	(III)									A	B	C		
2	1/2c	I	II	(III)										B		D	
3	3a		II	(III)	IV								A	B			
	3b		II	(III)	IV							(XII	XIII)	A	B	C	
	3c		II	(III)	IV							(XII	XIII)	A	B	C	
4	4a			(III)		(V)		VII		IX				A	B	C	
	4ab			(III)		V	VI	VII		IX	X			A	B	C	
	4b[d]			(III)		V	VI							A	B	C	
	4c			(III)		V		VII						A	B	C	
	4d			(III)		(V)	VI		VIII					A	B	C	
	4e			(III)		V	VI		(VIII)	(IX)				A	B	C	
	5			(III)		(V)	VI		(VIII)		X			A	B	C	
	6a			(III)		V	(VI)	(VII)		(IX)	X			A	B	C	
	6b			(III)		(V)		(VII)		IX	X	XI		A	B	C	
L. grayi				(III)								XII	XIV				E
L. murrayi				(III)								XII	XIV				E

[a] Paterson, 1940
[b] Seeliger, 1958
[c] Donker-Voet, 1959
[d] Also 4b(x): [(III) V VI VII] (McLauchlin et al., 1989)

elderly and immunocompromised (Lamont et al., 1988; Gellin and Broome, 1989). Albritton et al. (1984), analyzing the cases of listeriosis reported to the Centers for Disease Control, Atlanta, GA, found that most occurred in the very young or the elderly, with cases of neonatal listeriosis accounting for the largest recognizable group of patients.

Foodborne Outbreaks. L. monocytogenes has been identified as the cause of several outbreaks of foodborne disease (Table 1-2). The first outbreak where L. monocytogenes of the epidemic serotype was isolated from the implicated food, occurred in the Canadian Maritime Provinces in 1981 (Schlech et al., 1983; Schlech, 1984). There were a total of 17 deaths among the 41 cases, a mortality rate of 41%. After coleslaw consumption was associated with illness in epidemiological studies, coleslaw obtained from the refrigerator of one of the patients was shown to contain L. monocytogenes type 4b, the epidemic strain. A single plant had distributed this coleslaw throughout the Maritime Provinces. A shipment of some 2,000 kg of cabbage raised in fields fertilized by manure from a flock of sheep with listeriosis, had been sold to the implicated coleslaw plant after several months cold-storage . As Listeria species are able to grow at temperatures as low as 0°C, the prolonged cold storage of the vegetable acted essentially as a period of selective enrichment for this species.

An outbreak of listeriosis occurred in eight Boston hospitals in the fall of 1979 amongst 20 adult patients, 10 of whom were immunocompromised due to chemotherapy or steroid treatment (Ho et al., 1986). Three patients (15%) died of infection by L. monocytogenes serotype 4b. Epidemiological studies tentatively implicated three foods associated with the infection, i.e., tuna fish, chicken salad, and cheese. These foods were the only ones served with a salad of celery, tomatoes and lettuce. The raw vegetables were no longer available for isolation of the organism, so their implication as the source of infection is based on epidemiological evidence only.

In the summer of 1983, an outbreak of listeriosis occurred among 49 patients in Massachusetts (Fleming et al., 1985), of whom 42 were immunocompromised adults and 7 were

Table 1-2. Foodborne outbreaks of L. monocytogenes infections

Year	Location	Implicated Food	Cases		Deaths		Mortality (%)
			Adult	Perinatal	Adult	Perinatal	
1979[a]	Boston, MA	Raw vegetables (?)	20	0	3	0	15
1981[b]	Maritime Prov.	Coleslaw	7	34	2	15	41
1983[c]	Massachusetts	Pasteurised milk	42	7	12	2	29
1985[d]	California	Jalisco cheese	49	93	18	30	34
1983-1988[e]	Vaud Canton, Switz.	Raw milk cheese	59	63	NK[f]	NK	25

[a] Ho et al., 1986
[b] Schlech et al., 1983
[c] Fleming et al., 1985
[d] Linnan et al., 1988
[e] Bille, 1990
[f] NK - not known (31 deaths were recorded, but breakdown into adult and perinatal cases is unclear)

perinatal cases; 14 patients (29%) died. The epidemic strain was L. monocytogenes serotype 4b. Epidemiological studies showed that illness was strongly associated with drinking a specific brand of pasteurized milk. The implicated milk came from a group of farms where bovine listeriosis was known to have occurred at the time of the outbreak. L. monocytogenes was isolated from raw milk from these farms, but never found in the pasteurized product. There was no evidence of improper pasteurization procedures at the plant. The authors suggested that L. monocytogenes cells may have survived the pasteurization treatment.

The outbreak which raised listeriosis to a higher level of concern among food manufacturers and regulatory agencies (Wehr, 1987) occurred in California in 1985 (Linnan et al., 1988). There were 142 cases (93 perinatal and 49 adult), of whom 48 died (34%). The deaths included 30 foetuses and new born infants, and 18 immunosuppressed or elderly adults Case-control studies implicating Jalisco brand soft cheese were confirmed by laboratory studies which identified the presence of L. monocytogenes serotype 4b, of the epidemic phage type, in this cheese. Environmental samples taken from the plant producing the implicated cheese were positive for the organism of the epidemic phage type. The factory was closed and all the cheese of that brand recalled - the first recorded listeriosis outbreak in which the food causing the epidemic was identified and recalled during the outbreak

Over a period of several years (1983-1988), 122 cases of listeriosis with 31 deaths (28%) were associated with the consumption of raw milk soft cheeses manufactured in Vaud Canton, Switzerland (Anon, 1988; Bille, 1990). The cheeses were withdrawn from both the local and export market.

A survey, conducted by the Centers for Disease Control, Atlanta, GA, during 1986-87, showed that one-third of 154 cases of L. monocytogenes infection occurring in the United States were perinatal, and the remainder occurred in elderly or immunocompromised adults, with an overall mortality rate of 28% (Schwartz et al., 1988). Eighty-two cases and 239 controls were studied for risk factors for listeriosis, revealing that cases were significantly more likely than

controls to have eaten uncooked hot dogs or undercooked chicken, with 20% of the overall risk of listeriosis attributed to consumption of these foods. No other risk factors were identified.

Recently, it has been proposed that the risk factors in listeriosis, such as certain food products, may be associated with asymptomatic carriage of L. monocytogenes, and that the transition to invasive disease may be due to an enteric infection by a coinfecting organism, such as Salmonella spp. (Cox, 1989). In an outbreak of 36 cases of listeriosis in Philadelphia from December, 1986 to March, 1987, no predominant strain of the organism was found nor was the organism isolated from the foods identified as risk factors in case-control studies (Schwartz et al , 1989). Both authors hypothesized that the increase in cases of listeriosis may therefore result from an increase in infections by Salmonella or other enteric organisms.

Incidence of L. monocytogenes in Foods. As the number of reported cases of listeriosis with its high mortality rate and its clear-cut implication as a foodborne disease increased, the incidence of L. monocytogenes in foods became the subject of close scrutiny by both food manufacturers and food regulatory agencies (Hird, 1987; Farber and Losos, 1988; Brackett, 1988; WHO Working Group, 1988). The incidence rates reported in dairy products and raw vegetables in North America and Europe were generally low, ranging from 0 to 12% (Table 1-3), the exception being the presence of the organism in 45% of raw milk samples analyzed in Spain. The situation is different for meats and seafood products. In these foods, incidence rates for L. monocytogenes vary from 5 to 86%. This high incidence is of concern as some of these foods, such as dry-cured sausage and precooked poultry, are eaten with no further cooking. L. monocytogenes was found in 22/634 (3.5%) environmental samples taken from 17 food manufacturing facilities in Europe (Cox et al. 1989). It was also found in 5/105 (4.8%) domestic environmental samples.

The consumption of food contaminated with L. monocytogenes may therefore be quite common. Improved food sanitation with regard to Listeria spp., and more rapid and specific detection methods for the organism in foods, are both required so that the risk of infection by this organism via the oral route can be reduced.

Table 1-3. Incidence of L. monocytogenes in foods

Food	Location	No. positive / No. analyzed	Incidence (%)	Ref.
Raw milk	Ontario	17/315	5	Slade et al., 1988
	Ontario	6/455	1	Farber et al., 1988a
	Manitoba	4/256	2	Davidson et al., 1989
	Ohio	13/350	4	Lovett et al., 1987
	California	0/100	0	"
	Massachusetts	14/200	7	"
	Massachusetts	15/121	12	Hayes et al., 1986
	Nebraska	8/200	4	Liewin and Plautz, 1988
	Scotland	7/180	4	Fenlon and Wilson, 1989
	Spain	43/95	45	Dominguez et al., 1985
Pasteur. milk	Ontario	0/14	0	Farber et al., 1989
Cheese	Ontario	2/374	1	Farber et al., 1987
	Switzerland	50/1004	5	Breer and Schopfer, 1988
Frozen dairy prod.	Ontario	2/520	1	Farber et al., 1989
Raw vegetables	Ontario	0/110	0	Farber et al., 1989
	England	5/120	4	Sizmur and Walker, 1988
Dry-cured sausage	Canada	5/96	5	Farber et al., 1988d
	Ontario	6/30	20	Farber et al., 1989
	France	33/480	7	Leguilloux et al., 1980
Ground meat	Ontario	38/44	86	Farber et al., 1989
	Denmark	6/51	12	Skovgard and Norrung, 1989
Poultry (raw)	Ontario	9/16	56	Farber et al., 1989
Poultry (precooked)	England	63/527	12	Gilbert et al., 1989
Frozen seafood	9 countries (US imports)	15/57	26	Weagant et al., 1988

Thermal resistance of the Organism. The question of whether L. monocytogenes is more resistant to pasteurization than other nonsporing foodborne pathogens arose following the 1983 Massachusetts outbreak. Epidemiological evidence suggested that pasteurized milk was the vehicle of infection in this outbreak, though no evidence of faulty pasteurization was found in the implicated dairy. It was suggested at that time that the location of the organism within leukocytes provided a protective milieu (Fleming et al., 1985).

Conflicting results were published on the thermal resistance of L. monocytogenes cells in a variety of dairy products. Doyle's group (Doyle et al., 1985; Ryser et al., 1985) reported that L. monocytogenes inoculated into skim milk at a level of $1x10^5.mL^{-1}$, survived a heating process of 57.2°C for 30 min. When milk containing L. monocytogenes (Scott A) within polymorphonuclear leukocytes (PMNL) was heated to 71.7°C for 15s (high-temperature, short-time (HTST) pasteurization conditions) the intracellular organisms survived (Doyle et al., 1987). Garayzabal et al. (1986) also reported the survival of the organism in 6/28 (21%) samples of pasteurized (78°C for 15 s) naturally-contaminated milk marketed by a Madrid dairy. Bunning et al. (1988) determined that L. monocytogenes cells in whole milk can survive 71.7°C for 15s, whereas 62.8°C for 30 min yielded a safe margin of inactivation. The results were similar whether the cells were located extra- or intracellularly. Garayzabal et al. (1987) showed that though no L. monocytogenes cells were isolated from whole milk immediately after pasteurization at temperatures ranging from 69° to 72°C for 15s, the organism could be isolated from these same samples after storage at either 4°C or -28°C over a 3 week period.

In contrast to these reports of thermal resistance in L. monocytogenes cells, Bradshaw et al. (1985, 1987) reported that the Scott A strain suspended in raw milk, cream or ice cream mix and heated in sealed glass tubes did not survive 71.7°C for 15s. Farber et al. (1988c) reported that no viable L. monocytogenes were recovered from raw milk after treatment at 69°C for 16.2s. The bacteria were both extra-and intracellularly located, and were detected by both a 3-tube most-probable-number (MPN) technique using a 24 h resuscitation at 30°C, and a cold

enrichment technique with a sampling period up to 2 mo. Recent appreciations of the problem (Doyle, 1988; Farber, 1989) show that there is still disagreement on the question of an enhanced thermal resistance of L. monocytogenes. It was suggested by these authors that differences in the results from various studies were due to: a) different recovery procedures on stressed organisms, b) location of the organism within macrophages or PMNL, and c) initial numbers of the organism. As most dairies run their pasteurizers well above the minimum HTST conditions, the conclusion is that milk is generally safe for human consumption However, the survival of the organism in heat-treated and raw milk cheeses should be of concern to the dairy industry and to health officials.

1.1.3 Hemolysins of L. monocytogenes

Both the hemolytic activity of L. monocytogenes and its capacity for intracellular growth are recognized as components of the virulence of this organism (Rocourt and Seeliger, 1987; Cossart and Mengaud, 1989). Secretion of the hemolysin has been demonstrated as a major mechanism promoting intracellular growth of L. monocytogenes (Berche et al., 1987, 1988, Tilney and Portnoy, 1989). The pathogenicity of Listeria spp. isolates is usually tested by animal inoculation, especially intraperitoneal injection in mice (Mackaness, 1962; Stelma et al., 1987) Mainou-Fowler et al. (1988) showed that the hemolytic species, L. monocytogenes and L. ivanovii, had mouse LD_{50} values in the range of 0.4-40 X 10^6 cfu, and grew for 2-3 days within macrophages. The non- or weakly-hemolytic L. innocua, L. welshimeri and L. seeligeri, on the other hand, had LD_{50} values $>10^9$ cfu, were eliminated early, and inflicted minimal histological change. Three strains of L. monocytogenes grown at a reduced temperature (4°C) showed an increase in virulence for intravenously, but not intragastrically, inoculated mice, compared to the same strains grown at 22°C (Czuprynski et al., 1989).

As hemolytic activity may correlate with the pathogenicity of L. monocytogenes, several in vitro procedures demonstrating hemolysis have been proposed as a rapid alternative to the

mouse inoculation test. The simplest test for the presence of ß-hemolysis is the appearance of a zone of clearing on horse blood agar plates. However, the significance of these zones is often difficult to interpret, as strains of L. monocytogenes and L. seeligeri vary in the intensity of their reaction. A microplate technique proposed by Dominguez Rodriguez et al. (1986) for the routine determination of hemolytic activity against sheep erythrocyte suspensions, is a reliable method yielding semi-quantitative results. Continuous cell lines have also been used for testing hemolytic Listeria spp. Farber and Speirs (1987a) found that Chinese hamster ovary (CHO) cells were the most sensitive of 8 cell lines tested, in demonstrating a cytotoxic activity in culture supernatants of 5 Listeria spp. L. monocytogenes and L. ivanovii showed cytolysis, whilst L. innocua, L. murrayi and L. grayi did not.

Groves and Welshimer (1977) used the results of three in vitro reactions to identify pathogenic Listeria spp. - a positive CAMP reaction, fermentation of rhamnose and non-fermentation of xylose. The CAMP reaction was considered to be positive when there was an enhancement of the zone of hemolysis within an S. aureus hemolytic zone on sheep blood agar. Using these tests, the authors reported that the pattern of reactions correlated with the pathogenicity of 112 L. monocytogenes strains as tested by animal inoculation. The testing of Listeria spp. for the CAMP reaction was improved by Skalka et al. (1982) by using horse blood agar, and by the addition of a streak of Rhodococcus equi , which enabled L. ivanovii to be differentiated from L. monocytogenes and L. seeligeri by their opposite reactions with the hemolysins of the two test organisms.

Biochemistry of hemolysins. The ß-hemolysin, designated listeriolysin O (Geoffroy et al., 1987) by analogy with streptolysin O (SLO), was first isolated from L. monocytogenes culture supernatants and shown to be an SH-activated cytolysin sharing properties with SLO (Njoku-Obi et al., 1963). All strains of L. monocytogenes examined by Geoffroy et al. (1989) produced listeriolysin O. L. ivanovii and L. seeligeri strains also produced thiol-dependent exotoxins, at

about 10 times and 1/10 the level respectively, as that found in L. monocytogenes. Hemolysin was not found by these workers in L. innocua or in L. welshimeri strains.

Listeriolysin O was purified by Geoffroy et al. (1987, 1988) using thiol-disulfide exchange affinity chromatography. The hemolysin separated as a homogeneous protein with a MW of 60 kDa, and showed antigenic cross-reactivity with SLO. The lytic activity was inhibited by cholesterol and oxidizing agents in common with other SH-cytolysins, and activated by reducing agents such as thiols. The in vitro inactivation of listeriolysin O by cholesterol is thought to be due to competitive binding at the membrane binding site of the hemolysin, in common with other SH-activated cytolysins (Tanycz and Ziegler, 1988). There is evidence that different domains are involved in cytolytic activity and cholesterol binding. A 52 kDa truncated listeriolysin O lacking a 48 amino acid C-terminal oligopeptide containing the essential cysteine residue, lacked hemolytic activity but still bound to the membrane receptor cholesterol (Vazquez-Boland et al., 1989a). Listeriolysin O differed from the other SH-activated toxins (eg.pneumolysin, perfringolysin, alveolysin, SLO), however, in that it had a lower optimum pH of 5.5 for activity, and was reversibly inactived at pH 7.0 (Geoffroy et al., 1988). The SH-dependent hemolysins of L. ivanovii and L. seeligeri, both with MW of about 60 kDa, were shown tc be antigenically related to the L. monocytogenes listeriolysin O by Western blot analysis (Geoffroy et al., 1989; Vazquez-Boland et al., 1989b).

Hemolysins from L. monocytogenes and L. ivanovii were purified to homogeneity, characterized and sequenced (Goebel et al., 1988a, 1988c; Kreft et al., 1989). The purified proteins (MW 58 kDa) were activated by SH-reagents, inhibited by cholesterol, and they cross-reacted with SLO antibodies. Hof and Hefner (1988) in a report on the pathogenicity of Listeria spp., demonstrated that only L. monocytogenes and L. ivanovii, each of which possesses the ß-hemolysin, were able to multiply within mice after intravenous injection. Strains of the non-hemolytic L. innocua and L.welshimerii were avirulent. L. seeligeri, though weakly hemolytic, was also avirulent.

There have been several reports indicating the presence of more than one hemolysin in Listeria species. Listeriolysin O from L. ivanovii with a MW of 60 kDa, was used to prepare antibodies for immunoblotting of membrane-bound listeriolysin (Parrisius et al., 1986). Of the 28 ß-hemolytic L. monocytogenes strains tested in this way, only 2 produced a positive reaction. This unexpected result suggested the production of at least two immunologically distinct ß-hemolysins by L. monocytogenes strains. Kreft et al. (1989) reported that a second protein (MW 24 kDa) copurified with the ß-hemolysin of L. ivanovii, and was separated from it by gel filtration in the presence of SDS. This smaller protein was strongly hemolytic against sheep erythrocytes when combined with culture supernatants from R. equi, and not, when combined with supernatants from S. aureus. It may therefore represent the CAMP factor. There have been two reports of L. monocytogenes gene banks containing clones for more than one hemolysin determinant (Leimeister-Wächter et al., 1987; Vicente et al., 1987a). These will be discussed below in the section on Genetics of hemolysins.

Determination of the N-terminal sequences of the 58 kDa and the 24 kDa proteins showed no homology with the N-termini of other SH-activated cytolysins. Listeriolysin O is secreted by all virulent strains of L. monocytogenes, but it could not be demonstrated in the supernatants of L. innocua, L. seeligeri, L. welshimeri, L. grayi and L. murrayi by cross-reaction with anti-listeriolysin O or anti-SLO antibodies (Goebel et al., 1988c).

It was not possible to demonstrate a direct relationship between virulence and the amount of hemolysin produced. Using a hyperhemolytic (Hly^{++}) strain of L. monocytogenes, Kathariou et al. (1988) showed increased levels of production of the 58 kDa-hemolysin in the Hly^{++} strain. In spite of the increased hemolysin production, virulence as measured by a) number of cells required to infect, b) number of cells isolated from the spleen during infection, and c) time course to death, remained unchanged. Similarly, differences in virulence induced by growth of the organism at a reduced temperature (4°C), were not reflected in the amount of hemolysin released (Czuprynski et al., 1989).

Genetics of hemolysins. In further attempts to identify the role of hemolysin in the virulence of L. monocytogenes, transposon mutagenesis using the tetracycline resistance transposon Tn916 was used to inactivate the genetic determinant for hemolysin production (Kathariou et al., 1987a, 1987b). Three nonhemolytic (Hly^-) transconjugants found by screening, and a randomly-selected hemolytic (Hly^+) transconjugant, were chosen for further study. The Hly^- mutants were found to be avirulent in the mouse infection test (Mackaness, 1962), in contrast to the parent strain and the Hly^+ mutant. Though the Hly^- mutants were taken up by the mouse spleen cells, they failed to multiply, and were eliminated from the animals within 1 day. The Hly^- mutants lacked the 58 kDa extracellular protein (listeriolysin O); two produced a truncated protein of 49 kDa. Hly^+ revertants regained the hemolytic phenotype, and demonstrated virulence, and production of the 58 kDa extracellular protein (Kathariou et al., 1987b). Gene complementation was used to demonstrate the absolute requirement for listeriolysin O in the virulence of the organism (Cossart et al., 1989). A nonhemolytic, avirulent mutant was transformed with a plasmid carrying only hlyA on its insert. The complemented strain displayed a hemolytic phenotype and was fully virulent, thus showing that the loss of virulence in transposon-derived nonhemolytic mutants was not due to a polar effect in genes adjacent to hlyA.

Further studies on the uptake and intracellular growth of Kathariou's Tn916-induced Hly mutants were performed using invasion assays with mouse peritoneal macrophages, and mouse embryonic fibroblasts (Goebel et al., 1988b; Kuhn et al., 1988). Survival, but not uptake, of the bacterial cells was reduced significantly in the hemolysin-deficient mutants compared to hemolytic L. monocytogenes strains. Strains of other Listeria species, including the hemolytic avirulent L. seeligeri, and the strongly hemolytic L. ivanovii, did not penetrate the mouse fibroblast cells, even though L. ivanovii is pathogenic in mice. Uptake of the L. monocytogenes was abolished by treatment of the mouse fibroblast cells by cytochalasin B, a drug which inhibits microfilament function and hence endocytosis. Kuhn et al. (1988) concluded that the hemolysin is required for the intracellular survival of the organism, but not its initial entry. This agrees with the report by

Gaillard et al. (1987), that hemolysin is a major factor in promoting intracellular growth. Cells of a hly⁻ mutant obtained by transposon mutagenesis were normally phagocytized by cells of a human colon carcinoma cell line but showed little or no intracellular growth. These authors suggested that disruption of the vacuole membranes by the hemolysin was a key mechanism in allowing intracellular growth. The requirement of hemolysin for intracellular growth was confirmed by Portnoy and his co-workers (Camilli and Portnoy, 1988; Portnoy et al., 1988) who developed transposon Tn916 mutants which were non-hemolytic, lacked a secreted 58 kDa protein and were avirulent. These mutants were defective in intracellular growth. Revertants were hemolytic, secreted the 58 kDa protein, were virulent and able to grow intracellularly. The change of a common bacterium into a virulent organism was observed by Bielecki et al. (1990) when the hlyA gene was cloned into Bacillus subtilis host cells. These cells then expressed hemolysin and were able to grow intracellularly. These studies have amply demonstrated that the ß-hemolysin is an essential virulence factor of the organism, and the only bacterial gene product known to be absolutely required for intracellular growth (Cossart and Mengaud, 1989).

Transposon mutagenesis has been used to prepare Hly⁻ mutants useful in studying the sequence of the hemolysin determinant of this organism (Gaillard et al., 1986). The conjugative 26kb transposon Tn1545 encoding kanamycin, tetracycline and erythromycin resistance, was transferred to L. monocytogenes NCTC 7973, a hemolytic virulent strain, with a frequency of 10^{-8}. The resulting nonhemolytic mutant also showed loss of virulence in mice. The ability to infect mice, and to grow in spleen and liver cells, was restored by spontaneous loss of the transposon. The Hly⁻ mutant secreted a truncated protein of 52 kDa, which was detected by immunoblotting with an antiserum raised against listeriolysin O, thus demonstrating the insertion of Tn1545 in the structural gene for this protein. The insertion region of the transposon was then cloned and sequenced (Fig. 1-1; Mengaud et al., 1987, 1988a, b). The transposon had inserted in an open reading frame (ORF). The deduced amino acid sequence (Fig. 1-2; Mengaud et al., 1988b) of this ORF revealed homology with streptolysin O and pneumolysin (Kehoe and Timmis, 1984). DNA-

DNA hybridization showed that this hlyA sequence is present only in L. monocytogenes in the genus Listeria (Mengaud et al., 1988b; Cossart,1988).

The hlyA gene region has been studied to learn how the gene is regulated, and if silent copies of the gene exist in non-hemolytic species. The 5' adjacent regions have sequences which show homology to L. ivanovii and L. seeligeri, but the downstream regions appear specific to L. monocytogenes (Gormley et al., 1989). A spontaneous 300 bp deletion located 1.6 kb upstream from an intact hlyA gene resulted in the production of a non-hemolytic, avirulent mutant indicating an area involved in controlling the expression of the gene (Leimeister-Wächter et al., 1989). Sequence analysis of the gene region revealed the presence of two ORFs: ORF D located downstream from hlyA, and ORF U located upstream and in the opposite direction (Mengaud et al., 1989). hlyA and ORF U are transcribed in opposite directions from promoters which are adjacent. These two promoter regions are separated by a 14-bp palindromic sequence. This palindrome was also found upstream of the ORF D promoter, suggesting that all three genes are similarly regulated.

In the first reported cloning of L. monocytogenes genomic material, a gene bank of L. monocytogenes DNA partially digested with Mbo I, was prepared in the cosmid vector pHC79 and used to transform E. coli HB101 cells (Vicente et al., 1985). Twelve clones expressing hemolysin were identified, with inserts of about 35 kb. The size difference between the 6 kb vector and the insert, resulted in unstable recombinants from which the cloned fragments were easily lost.

Gene banks of L. monocytogenes DNA have produced evidence for the existence of more than one hemolysin in this organism (Leimeister-Wächter et al., 1987; Vicente et al., 1987a). Approximately 10,000 clones of L. monocytogenes 1/2a strain gene bank in E. coli were screened, of which 1 was identified as a hemolytic phenotype, expressing a 23 kDa protein which did not cross-react with anti-streptolysin O antibodies (Chakraborty et al., 1986; Leimeister-Wächter et al., 1987). In a further report by Vicente et al. (1987a), one of their clones containing a 17kb insert was partially deleted between BamH I and Sall sites, resulted in a stable hemolysin-positive clone

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AGCAAGCATATAATATTGCGTTTCATCTTTAGAAGCGAATTTCGCCAATATTATAATTATCAAAAGAGAGGGGTGGCA

AACGGTATTTGGCATTATTAGGTTAAAAAATGTAGAAGGAGAGTGAAACCCATGAAAAAAATAATGCTAGTTTTTATT
                                    SD                  M  K  K  I  M  L  V  F  I
79                                                                                9
ACACTTATATTAGTTAGTCTACCAATTGCGCAACAAACTGAAGCAAAGGATGCATCTGCATTCAATAAAGAAAATTCA
 T  L  I  L  V  S  L  P  I  A  Q  Q  T  E  A  K  D  A  S  A  F  N  K  E  N  S
157                                                                              35
ATTTCATCCATGGCACCACCAGCATCTCCGCCTGCAAGTCCTAAGACGCCAATCGAAAAGAAACACGCGGATGAAATC
 I  S  S  M  A  P  P  A  S  P  P  A  S  P  K  T  P  I  E  K  K  H  A  D  E  I
235                                                                             61
GATAAGTATATACAAGGATTGGATTACAATAAAAACAATGTATTAGTATACCACGGAGATGCAGTGACAAATGTGCCG
 D  K  Y  I  Q  G  L  D  Y  N  K  N  N  V  L  V  Y  H  G  D  A  V  T  N  V  P
313                                                                             87
CCAAGAAAAGGTTACAAAGATGGAAATGAATATATTGTTGTGGAGAAAAAGAAGAAATCCATCAATCAAAATAATGCA
 P  R  K  G  Y  K  D  G  N  E  Y  I  V  V  E  K  K  K  K  S  I  N  Q  N  N  A
391                                                                            113
GACATTCAAGTTGTGAATGCAATTTCGAGCCTAACCTATCCAGGTGCTCTCGTAAAAGCGAATTCGGAATTAGTAGAA
 D  I  Q  V  V  N  A  I  S  S  L  T  Y  P  G  A  L  V  K  A  N  S  E  L  V  E
469                                                                            139
AATCAACCAGATGTTCTCCCTGTAAAACGTGATTCATTAACACTCAGCATTGATTTGCCAGGTATGACTAATCAAGAC
 N  Q  P  D  V  L  P  V  K  R  D  S  L  T  L  S  I  D  L  P  G  M  T  N  Q  D
547                                                                            165
AATAAAATCGTTGTAAAAAATGCCACTAAATCAAACGTTAACAACGCAGTAAATACATTAGTGGAAAGATGGAATGAA
 N  K  I  V  V  K  N  A  T  K  S  N  V  N  N  A  V  N  T  L  V  E  R  W  N  E
625        Hind III                                                            191
AAATATGCTCAAGCTTATCCAAATGTAAGTGCAAAAATTGATTATGATGACGAAATGGCTTACAGTGAATCACAATTA
 K  Y  A  Q  A  Y  P  N  V  S  A  K  I  D  Y  D  D  E  M  A  Y  S  E  S  Q  L
703                                                                            217
ATTGCGAAATTTGGTACAGCATTTAAAGCTGTAAATAATAGCTTGAATGTAAACTTCGGCGCAATCAGTGAAGGGAAA
 I  A  K  F  G  T  A  F  K  A  V  N  N  S  L  N  V  N  F  G  A  I  S  E  G  K
781                                                                            243
ATGCAAGAAGAAGTCATTAGTTTTAAACAAATTTACTATAACGTGAATGTTAATGAACCTACAAGACCTTCCAGATTT
 M  Q  E  E  V  I  S  F  K  Q  I  Y  Y  N  V  N  V  N  E  P  T  R  P  S  R  F
859                                                                            269
TTCGGCAAAGCTGTTACTAAAGAGCAGTTGCAAGCGCTTGGAGTGAATGCAGAAAATCCTCCTGCATATATCTCAAGT
 F  G  K  A  V  T  K  E  Q  L  Q  A  L  G  V  N  A  E  N  P  P  A  Y  I  S  S
937                                                                            295
GTGGCGTATGGCCGTCAAGTTTATTTGAAATTATCAACTAATTCCCATAGTACTAAAGTAAAAGCTGCTTTTGATGCT
 V  A  Y  G  R  Q  V  Y  L  K  L  S  T  N  S  H  S  T  K  V  K  A  A  F  D  A
1015                                                                           321
GCCGTAAGCGGAAAATCTGTCTCAGGTGATGTAGAACTAACAAATATCATCAAAAATTCTTCCTTCAAAGCCGTAATT
 A  V  S  G  K  S  V  S  G  D  V  E  L  T  N  I  I  K  N  S  S  F  K  A  V  I
1093                                                                           347
TACGGAGGTTCCGCAAAAGATGAAGTTCAAATCATCGACGGCAACCTCGGAGACTTACGCGATATTTTGAAAAAAGGC
 Y  G  G  S  A  K  D  E  V  Q  I  I  D  G  N  L  G  D  L  R  D  I  L  K  K  G
1171                                                                           373
GCTACTTTTAATCGAGAAACACCAGGAGTTCCCATTGCTTATACAACAAACTTCCTAAAAGACAATGAATTAGCTGTT
 A  T  F  N  R  E  T  P  G  V  P  I  A  Y  T  T  N  F  L  K  D  N  E  L  A  V
1249                                Hind III                                   399
ATTAAAAACAACTCAGAATATATTGAAACAACTTCAAAAGCTTATACAGATGGAAAAATTAACATCGATCACTCTGGA
 I  K  N  N  S  E  Y  I  E  T  T  S  K  A  Y  T  D  G  K  I  N  I  D  H  S  G
1327                                                                           425
GGATACGTTGCTCAATTCAACATTTCTTGGGATGAAGTAAATTATGATCCTGAAGGTAACGAAATTGTTCAACATAAA
 G  Y  V  A  Q  F  N  I  S  W  D  E  V  N  Y  D  P  E  G  N  E  I  V  Q  H  K
1405                                                                           451
AACTGGAGCGAAAACAATAAAAGCAAGCTAGCTCATTTCACATCGTCCATCTATTTGCCAGGTAACGCGAGAAATATT
 N  W  S  E  N  N  K  S  K  L  A  H  F  T  S  S  I  Y  L  P  G  N  A  R  N  I
1483                                                                           477
AATGTTTACGCTAAAGAATGCACTGGTTTAGCTTGGGAATGGTGGAGAACGGTAATTGATGACCGGAACTTACCACTT
 N  V  Y  A  K  E  C  T  G  L  A  W  E  W  W  R  T  V  I  D  D  R  N  L  P  L
1561                                                                           503
GTGAAAAATAGAAATATCTCCATCTGGGGCACCACGCTTTATCCGAAATATAGTAATAAAGTAGATAATCCAATCGAA
 V  K  N  R  N  I  S  I  W  G  T  T  L  Y  P  K  Y  S  N  K  V  D  N  P  I  E
1639                                                                           529
TAA
```

Fig. 1-1. Nucleotide sequence of L. monocytogenes hlyA gene. The Hind III restriction sites are shown. The single-letter code of the deduced amino-acid sequence is shown, with the putative 25-amino acid signal sequence being underlined. The proposed Pribnow boxes, and Shine-Delgarno sequences are underlined. Coordinates under the left or right end of each line correspond to the nucleotide sequence and the deduced peptide sequence, respectively (Mengaud et al., 1988b).

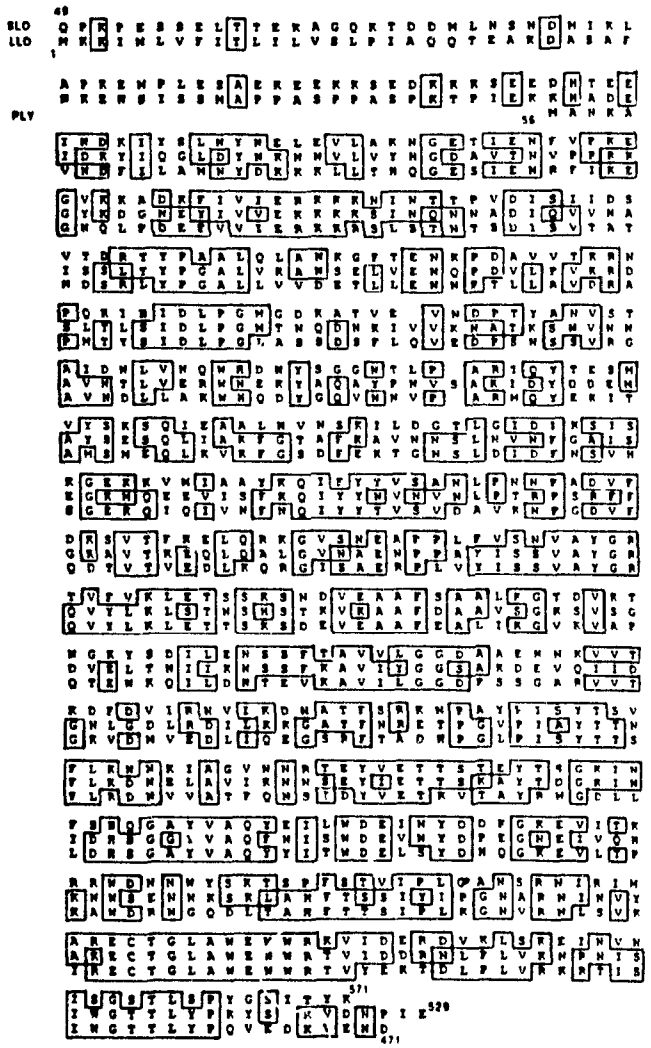

Fig. 1-2. Deduced amino acid sequence of listeriolysin O (L_O), aligned with streptolysin O (SLO) and pneumolysin (PLY). Identical amino acids have been boxed. The numbers indicate the amino acid coordinates (Mengaud et al., 1988b).

(pCL102) with an 8.3kb insert. Subcloning of Hind III fragments of pCL102 into pUC8 resulted in clones whose hemolytic activity was only detectable after sonication of the cells. Gel filtration of the sonicated preparation resulted in the elution of two peaks of hemolytic activity, corresponding to proteins of 22 kDa and 48 kDa, suggesting the existence of two hemolysins. The smaller protein, which was not thiol-activated, may be comparable to the 24 kDa protein isolated from L. ivanovii by Goebel et al. (1988c), suggested by them as the CAMP factor. It has been shown that the CAMP factor is not SH-activated (Kreft et al., 1989).

The genetic relatedness of the listeriolysin gene of L. monocytogenes to those of other Listeria spp. was demonstrated by Leimeister-Wachter and Chakraborty (1989). An L. monocytogenes gene bank in E. coli was prepared and clones that contained the hly determinant were identified by Western blotting with anti-listeriolysin O antiserum. Five DNA probes that encoded the listeriolysin gene and surrounding sequences were prepared from these clones. These were then used to detect homology to L. ivanovii and L. seeligeri. Homology was not detected towards L. innocua, L. welshimeri, L. grayi, or L. murrayi. However, Gormley et al. (1989) detected homology to the hlyA determinant in both virulent and avirulent hemolytic strains of several Listeria spp.

Conjugation experiments were carried out between Streptococcus agalactiae and three Listeria spp., demonstrating the possibility of genetic exchange among members of these genera (Perez-Diaz et al., 1982). Other evidence of conjugative transfer amongst members of the genera Listeria, Streptococcus, and Erysipelothrix has been reported (Flamm et al., 1984; Vicente et al., 1988). Both plasmids and transposons containing antibiotic resistance genes, were transferred, and the resistance determinants were expressed and stably inherited.

1.2 DNA Probes

Lengths of single-stranded DNA or RNA which carry specific nucleotide sequences in common, will, when mixed, anneal at complementary regions to form duplexes. This process of

duplex formation is called hybridization. The accuracy of the base-pairing during the annealing process can be affected by the stringency of the conditions under which it occurs, particularly the temperature. In general, hybridization and subsequent washing should be carried out at T_m-12°C where T_m is calculated from the equation (Maniatis et al., 1982):

$$T_m = 69.3° + 0.41(G+C)\% - (650/L)$$

where T_m = melting temperature of the DNA, G + C = the guanine plus cytosine content of the DNA, and L = the probe length in bp. Stringency can be maintained at a lower temperature by the use of formamide, which lowers the T_m of the duplex DNA by 0.7°C with each 1% increase in the formamide concentration. Further, the T_m of duplex DNA containing mismatched base pairs decreases by 1°C with each 1% increase in number of mismatches (Maniatis et al., 1982).

If one of the nucleic acid molecules in the duplex is labelled with either a radioisotope or a chromogen, it can be used to detect the presence of the complementary sequence. These labelled molecules, known as probes, can be prepared from cloned inserts as polynucleotides, or synthesized as oligonucleotide sequences. Internal labelling, by nick translation or random primed labelling, is used for labelling of longer cloned probes. End labelling, on the other hand, is used for the shorter synthetic probes.

The preparation of polynucleotide probes from inserts in large quantities is labour-intensive, the restriction fragments must be made free of vector sequences, and the probes need to be denatured for hybridization to occur. Single-stranded oligonucleotide probes, on the other hand, can be prepared synthetically in large quantities (Berent et al., 1985). Recently, it was demonstrated that synthetic oligonucleotide probes containing 30 or 33 bp, and polynucleotide probes, were equally efficient in the detection of E. coli, Vibrio cholerae and Yersinia enterocolitica (Sommerfelt et al., 1988).

1.2.1 Colony and Blot Hybridization

Hybridization has been used as a research tool in the testing of DNA relatedness by immobilizing isolated DNA on nitrocellulose (NCM) or nylon membranes, and detecting the nucleic acid with labelled DNA probes. DNA electrophoretically separated on an agarose or polyacrylamide slab gel can also be transferred to NCM by capillary or electrical action prior to DNA hybridization (Southern, 1975). In both cases, the DNA is denatured by treatment with NaOH and fixed to the nitrocellulose by baking or UV irradiation. After hybridization, sequence homology is detected using either autoradiography on X-ray film when probes are labelled by radioisotopes, or by the in situ deposit of a visible precipitate when probes are labelled by a chromogen. This technology was adapted as a means of detecting a specific sequence amongst thousands of cloned DNAs with the development of colony hybridization and its variant, blot hybridization, by Grunstein and Hogness (1975). The colonies to be screened are formed on, or blotted to, a cellulose or NCM filter and the cells, after lysis, are probed as described above.

Colony hybridization is often used in the screening of genomic libraries, which are collections of randomly-cloned fragments of genomic DNA in either a plasmid or viral vector, that encompass the entire genome of a given species. The number of transformants (N) required to yield a predetermined probability (P) of cloning the entire genome is given by the formula (Clarke and Carbon, 1976):

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where f = the fraction of the entire chromosome represented by the average insert size. For example, for a bacterium with a genome size of 5×10^3 kb, and an average fragment size of 10 kb, it would be necessary to screen about 2300 recombinants in order to provide a 99% probability of encompassing the entire genome.

Transformed cells are identified by the use of a detectable marker, usually an antibiotic resistance determinant, on the vector. The presence of a recombinant plasmid within the

transformed cell is demonstrated by using a second genetic marker on the vector, such as a second antibiotic resistance gene or the ß-galactosidase lacZ gene, with restriction sites where the DNA fragments are inserted and ligated, resulting in insertional inactivation of that marker (Dale and Greenaway, 1984). Insertional inactivation of the lacZ gene results in recombinant clones that can be detected visually. When combined with a lacZ deletion in the host cell strain, and the incorporation of the substrate 4-bromo-5-chloroindolyl-ß-galactoside (X-gal) in the plating medium, clones of transformants are coloured blue by the formation of an indigo derivative, with clones containing recombinant plasmids remaining colourless.

Efforts to improve the sensitivity of hybridization procedures include the amplification of the target by the polymerase chain reaction (PCR; Olive et al., 1988). After DNA containing the sequence of interest was amplified by many cycles of PCR, a ligand, such as biotin, bound to sequence-specific oligonucleotides, was incorporated to the amplified region by another cycle of PCR. The ligand was then detected by enzyme-labelled avidin, followed by the indicated substrate (Kemp et al., 1989).

1.2.2 Colorimetric Labelling of Probes

There are a number of limitations associated with the use of radioisotope-labelled DNA probes. The isotopes are expensive, and require special precautions in their use and disposal. A commonly used radioisotope, ^{32}P, has the additional inconvenience of a short, 14-day half-life, leading to the need to prepare and label fresh probe frequently. The use of radio-labelled probes in food manufacturing facilities would likely be poorly received by the public. Currently, there are several approaches that can be taken to non-radioactive labelling of DNA probes, such as: 1) the DNA is labelled with biotin and detected by enzyme-labelled avidin, 2) the DNA is bound to a linker and detected by an enzyme-labelled molecule binding to the linker, 3) the DNA is labelled with a hapten and detected by an enzyme-labelled antibody to the hapten, 4) the DNA is directly

labelled with an enzyme. The last step in all these procedures is an enzyme-substrate reaction yielding a product which forms a visible precipitate in situ.

The high affinity constant ($K_D=10^{-15}$) between the glycoprotein avidin, found in egg-white, and the vitamin biotin, has led to their use as a tool in molecular biology (Bayer and Wilchek, 1980). Biotin-labelled analogues of UTP and dUTP were synthesized and used as substrates for RNA or DNA polymerases in nick-translation reactions (Langer et al., 1981; Leary et al., 1983). The biotin-labelled DNA probes so formed, when hybridized to target sequences immobilized on a membrane, were detected by the use of avidin labelled with an enzyme such as alkaline phosphatase (AP) or horseradish peroxidase (HRP). Coloured precipitates were developed at the sites of hybridization by placing the membrane in the appropriate substrate solution. The AP substrate was 5-bromo-4-chloro-3-indolyl phosphate (BCIP), whose product when combined with nitro blue tetrazolium (NBT), yielded a blue precipitate at the site of the reaction. The HRP substrate solution of H_2O_2 was used with 4-chloro-1-naphthol as the electron acceptor, yielding a dark purple precipitate at the reaction site. This method is available as a commercial kit, (Enzo Biochem, Inc.; Gardner, 1983).

A practical alternative to the labelling of DNA with biotin by enzymes such as DNA polymerase or terminal transferase, is a chemical biotinylation using photobiotin (McInnes et al., 1987). The probe DNA was mixed with N-(5-azido-2-nitrophenyl)-N'-(3-biotinylaminopropyl)-N'-methyl-1,3-propanediamine (photobiotin) and exposed to visible light, resulting in covalently bound biotin moieties on the DNA. After hybridization, the labelled DNA is detected by the methods described above. Probes labelled in this way were used in dot blot hybridizations to screen plasmids for a specific gene fragment with detection levels of 10 pg (Denman and Miller, 1989).

If single-stranded DNA is brominated, the bromine can subsequently be displaced by a primary amino group attached to a linker arm (Keller et al., 1988). This method was used to label a cloned DNA probe with 2,4-dinitrophenyl (DNP) attached to a linker arm of 1,6-diaminohexane.

After hybridization, the DNP was detected with a rabbit polyclonal anti-DNP antibody, followed by an AP-conjugated anti-rabbit second antibody. The detection sensitivity was 3 pg of target DNA in a dot blot on nitrocellulose. A photoactivable hapten was prepared by Keller et al (1989), 6-(2,4-dinitrophenylamino)-1-aminohexyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (photoDNP), which coupled efficiently to DNA when exposed to visible light. The probe was detected by the method described above. The procedure was able to detect 15 pg of a 12 kb target DNA.

Haptens can be introduced onto DNA probes and subsequently detected by enzyme-linked antibodies. A DNP derivative of ATP, was used both for 3'-end labelling of a DNA fragment by terminal transferase, and for nick translation labelling by DNA polymerase (Vincent et al., 1982) As well, chemical dinitrophenylation of 8-aminohexyl ATP can be carried out with 1-fluoro-2,4-dinitrobenzene, after incorporation of the ATP into DNA by either terminal transferase or DNA polymerase. These DNP-labelled DNAs were then detected, after hybridization, using rabbit anti-DNP antibody, followed by an HRP-conjugated anti-rabbit second antibody. Another hapten used in the development of immuno-detectable probes was N-acetoxy-N-2-acetylaminofluorene (AAF) which was attached to the guanine residues of DNA by iodination (Tchen et al, 1984), and detected by a monoclonal antibody raised against AAF-guanosine followed by a second antibody, AP- or HRP-conjugated anti-mouse IgG (Masse et al., 1985). This system was tested by Southern blot, colony and plaque hybridizations. Detection was at a level of 0.5 pg of target DNA; the probes were stable for a year at 4°C.

A commercial kit is available by which DNA is labelled with the hapten, digoxigenin (Dig), by random primed labelling. The label is detected by either an AP- or HRP-conjugated anti-Dig antibody (Boehringer-Mannheim, 1988). The method is applicable to Southern blot, dot blot, colony and plaque hybridizations, and can detect 1 pg of homologous DNA.

Renz and Kurz (1984) developed a method which allowed direct attachment of enzymes, such as AP or HRP, to single-stranded DNA. The protein is activated by benzoquinone and crosslinked to a low molecular weight polyethyleneimine (PEI). Linearized single-stranded probe

DNA was bound by electrostatic attraction to the PEI, and then cross-linked with glutaraldehyde. Using HRP-labelled DNA and 3,3'-anisidine as the oxygen acceptor, the authors were able to detect about 5 pg target DNA in blot and Southern hybridizations. This DNA probe labelling method using HRP-PEI was used in Southern blot (Downs et al., 1987) or colony hybridization (Romick et al., 1987) procedures. Methyl-2-benzothiazolinone hydrazone was used as the oxygen acceptor in the HRP colour development solution resulting in an improved detection limit of 1 pg for Southern blots (Downs et al., 1987). Colonies of enterotoxigenic E. coli grown on nitrocellulose membranes placed on the surface of agar plates, were detected by probes labelled with HRP (Romick et al., 1987). The labelled probes were reported to be stable for 3 mo at -20°C. The Renz and Kurz procedure was used to covalently link cytochrome c with attached biotin to a single-stranded DNA probe (Ziomek et al., 1987). Hybridization was detected using an HRP-labelled avidin, with a detection limit of 5 pg.

Synthetic oligodeoxynucleotides were directly labelled with AP by cross-linkage (Jablonski et al., 1986). One of the bases in the oligomer was modified with a 12-atom linker arm through which one oligomer was attached per enzyme molecule. Sequence homology was detected after hybridization by the formation of an insoluble enzyme-substrate product. Detection sensitivities for DNA dot blots on nitrocellulose varied from 20 pg to 50 ng.

1.2.3 Use In Detection of Foodborne Pathogens

Analytical food microbiology methods using DNA probes, have been reported for the following organisms: enterotoxigenic E. coli (ETEC) for both LT and ST, Salmonella spp., Yersinia enterocolitica, Vibrio parahaemolyticus, V. vulnificus, Campylobacter jejuni and L. monocytogenes. Of these the methods for LT and ST of E. coli, and for Salmonella spp. have been approved by the Association of Official Analytical Chemists (AOAC). The subject has been reviewed by Fitts (1986), Parsons (1988), and Wernars and Notermans (1990).

The use of DNA probes in analytical food microbiology was discussed by Hill (1981), who pointed out that a test for genotype has several advantages over one for phenotype. These advantages are: 1) organisms do not have to be isolated in pure culture, 2) expression of the gene is not required, 3) on occasion, a single probe can detect more than one virulence factor, e.g., LT and cholera toxin have sequences in common, 4) when working with isolates, large numbers (≈50) can be screened on a single filter. The disadvantages of testing for genotype are: 1) mutant genes may possess sequences complementary to a probe, but not express an active product, 2) the preparation of probes from clones requires time and skill, 3) viable cells must be retrieved from the product to be tested, 4) if the probe is radiolabelled, the test laboratory must be licenced, and equipped to handle radioactive isotopes safely.

Enterotoxigenic E. coli. A DNA colony hybridization method for the detection of enterotoxigenic E. coli (ETEC) was first applied to food products by Hill et al. (1983a). When tested with 18 strains of E. coli or ETEC, the results by colony hybridization were in agreement with the results obtained by enzyme-linked immunosorbent assay (ELISA), and the mouse Y-1 adrenal cell reaction. Food (scallops) artificially contaminated with ETEC was spread-plated onto nitrocellulose filters on agar plates and tested by DNA colony hybridization after incubation, with a recovery of 87%. In a collaborative study, 13 laboratories each tested 25 unknown and 3 control samples for the presence of the LT gene using a radiolabelled probe (Hill and Payne, 1984). Of the 325 samples 315 (97%) were identified correctly, with 6 false negative and 4 false positive identifications.

There were some disadvantages to this method such as the labour-intensive preparation of the DNA probe by the purification of cloned gene fragments, and the use of NCM filters which are costly, fragile, and may become brittle. With the availability of synthetic probes, it became possible for laboratories lacking personnel skilled in DNA techniques to use DNA probes. A study in which a 22-base ^{32}P-labelled oligomer for each of 2 E. coli ST genes, STH and STP, was prepared and sent to 23 collaborating laboratories resulted in 440 of the 460 unknown samples

(95.7%) being correctly identified (Hill et al., 1985). Filter paper, rather than NCM, was used as the solid support for the hybridization reaction.

A radioisotope-labelled cloned LT probe was used for the detection and enumeration of an LT-producing strain of E. coli in a variety of food products (Ferreira et al., 1986). The foods, artificially-inoculated with E. coli H10407 at levels of 4×10^2 to 3×10^3 cfu.mL^{-1}, were inoculated onto NCM, and incubated on plate count agar (PCA) and eosin methylene blue (EMB) plates. After hybridization and autoradiography, each dark spot on the X-ray film was counted as one organism. Recoveries of inoculated organisms were about 81% on PCA and 76% on EMB (ρ=0.58). There was no significant effect of food type on recoveries.

An experimental system was used by Dovey and Towner (1989) to evaluate a biotin-labelled DNA probe in artificially-contaminated food products. The probe, to the ß-lactamase gene in E. coli (pBR322), was labelled by nick-translation with biotin-11-dUTP, and used in a dot-blot hybridization technique with detection by an AP-avidin conjugate. The colour intensity of the product was assessed by a computer-controlled image analysis system. The method detected cells at a concentration of $\approx 10^7$ cells.g^{-1} food.

Salmonella serovars. Flowers (1985) discussed the need for practical, rapid Salmonella detection methods as compared to the lengthy conventional culture method. A DNA hybridization detection method for Salmonella spp. in foods, described by Fitts et al. (1983), required a pre-enrichment of food samples. Decimal dilutions of a variety of food products in nutrient broth (10 mL) were artificially inoculated with 10^3 salmonellae and incubated overnight at 37°C. Samples (1 mL) were removed and collected on nitrocellulose membranes by filtration. The DNA of the lysed cells was denatured and fixed, and then hybridized to radiolabelled probes. The probes consisted of DNA sequences from an S.typhimurium genomic library, which had been identified as being unique to Salmonella spp. (Fitts, 1983). There was no effect of food type on the selectivity or sensitivity (40 organisms.g^{-1} of food) of the method. It was later demonstrated that a selective enrichment step was required to raise the cell number to the level of $\approx 10^7$ cells.mL^{-1}

required for detection (Flowers, 1985). When 5 DNA probes were tested against 181 Salmonella serovars, including S. arizonae (450 strains in all), it was shown that a pool of the probes hybridized to all the test strains (Fitts, 1986). These probes formed the first commercial DNA probe for the detection of foodborne pathogens (Gene-Trak; Curiale et al., 1986). The commercial DNA hybridization method required about 44h to complete the steps of preenrichment, selective enrichment, hybridization, and ß-particle counting. This compares to the 4 days the conventional culture method requires to a negative result, with an additional 2 or 3 days to a confirmed positive result. More than 1600 samples, representing 23 different foods, and with 20 Salmonella serovars, were compared by the two methods (Flowers et al., 1987). Overall, the DNA hybridization method had 5.3% false-positive, and 1.8% false-negative results, compared to the AOAC conventional method.

Yersinia enterocolitica. Not all strains of Yersinia enterocolitica are able to cause disease. Several possible virulence factors have been suggested, many of which correlate with the presence of a 42-megadalton plasmid. The expression of the factors encoded by this plasmid responds to temperature and calcium as extracellular signals (Hill et al., 1983b; Miller et al., 1989). DNA fragments of this plasmid were cloned and tested as colony hybridization probes to identify virulent Y. enterocolitica strains in pure culture, and in artificially-contaminated food samples. The results agreed with autoagglutination, and suckling mouse tests. As the virulence plasmid of Y. enterocolitica is easily lost during incubation in enrichment broth, the DNA colony hybridization method which does not require an enrichment step, may improve the recovery from foods of Y. enterocolitica cells still carrying the virulence plasmid, enabling the identification of virulent strains. A probe consisting of a cloned fragment encoding the calcium dependency region, was used to enumerate Y. enterocolitica in 11 artificially-contaminated foods (Jagow and Hill, 1986). Recoveries, averaging 86%, were not affected by the medium used nor the NCM filter, but were influenced by the number of background organisms. If the total microbial population in the food

was above 10^7 cfu.g^{-1}, then identification of Y. enterocolitica by colony hybridization became difficult.

Other organisms. DNA probes for other foodborne pathogens have been described, but not yet reported as having been tested in foods. These include: a natural probe for the thermostable direct hemolysin of Kanagawa phenomenon-positive Vibrio parahaemolyticus (Nishibuchi et al., 1985); a synthetic probe for the same hemolysin (Nishibuchi et al., 1986); a natural probe for the cytotoxin-hemolysin of V. vulnificus (Morris, Jr., et al., 1987); the synthetic E. coli LT-B probe to detect Campylobacter jejuni (Baig et al., 1986); an AP-labelled synthetic probe for the detection of C. jejuni (Wong and Carito, 1989); a natural probe for enterohaemorrhagic E. coli (Levine et al., 1987).

The use of DNA probes for the detection of L. monocytogenes in foods will be discussed in Sect. 1.3.2.

1.3 Detection Methods for Listeria monocytogenes in Foods

The detection of low levels of L. monocytogenes in foods heavily contaminated with other organisms is difficult. The use of various agents including potassium tellurite, naladixic acid, acridine, and trypaflavine has been proposed to improve the selectivity of isolation media (Donnelly, 1988). However, these selective agents are detrimental to the recovery of cells injured by physical agents such as heat or irradiation (Curtis et al., 1989b). These authors recommended that selective media be incubated at 30°C, rather than 35°C, to improve the recovery of Listeria spp. Refrigeration of the sample in a nonselective medium for prolonged periods, up to 6 mo, has been used in the past to aid in the recovery of this psychrotrophic organism. McBride and Girard (1960) developed a selective agar medium which, coupled with the oblique illumination of the colonies suggested by Henry (1933), contributed to the successful isolations of L. monocytogenes from food. Many conventional culture methods have been developed recently (Donnelly, 1988; Cassiday and Brackett, 1989). Alternative methods using monoclonal antibodies (Farber et al.,

1988b) and DNA probes (Datta et al., 1987) offer better possibilities for rapid and specific detection of L. monocytogenes in foods.

1.3.1 Conventional Methods

Direct plating media. Many isolation media have been developed to recover Listeria spp. from foods. Direct plating procedures are not generally successful in isolating Listeria so they are used after a prior enrichment. A medium containing glycine anhydride and phenylethanol as selective agents, was developed by McBride and Girard (1960) for recovering L. monocytogenes from mixed cultures. Despierres (1971) medium contains nalidixic acid, polymyxin B and acriflavine as selective agents. A modification of McBride's agar (MMA; Lovett, 1988), containing phenylethanol, glycine anhydride, lithium chloride and cycloheximide as selective agents, is currently in use by the food industry and regulatory agencies. After incubation on MMA plates, Listeria spp. appear as small bluish granular colonies when the plates are examined by oblique (45°) transmitted light under a stereomicroscope (Henry, 1933). When the organisms are grown on transparent media, Henry's illumination is widely used to detect presumptive Listeria spp. A medium in which the agar was replaced by a self-gelling hydrocolloid gum allowed colonies of Listeria spp. to be readily identified by the use of oblique light (Martin et al., 1984). An improved selective medium was developed by Lee and McClain (1986) for the isolation of L. monocytogenes from meats. This medium was derived from Baird-Parker agar, and was the first to use moxalactam, a broad spectrum antibiotic inhibitory to many gram-positive and gram-negative bacteria. The medium, which contains lithium chloride, phenylethanol and moxalactam (LPM agar), improves the recovery of the organism from mixed cultures as compared to MMA.

Other formulations for plating media include: 1) Listeria selective agar (Dominguez medium; Dominguez Rodriguez et al., 1984); 2) the addition of moxalactam, bacitracin, nalidixic acid and potassium tellurite to Vogel Johnson agar (MVJ; Buchanan et al., 1987); 3) media developed by Mossel's group using acriflavine, lithium chloride and phenylethanol or antibiotics

as selective agents, and aesculin as a colour cue (RAPAMY and PALCAM agars; Van Netten et al., 1988a,b. 1989); 4) a medium based on Listeria enrichment broth (LEB agar; Donnelly and Baigent, 1986); 5) a selective agar (Oxford) with antibiotics and aesculin (Curtis et al., 1989a).

Loessner et al. (1988) compared 7 direct plating media, including the original McBride's agar, MMA, LPM agar, Dominguez medium and MVJ agar, for their suitability to enumerate Listeria spp. Their findings indicated: 1) all media were equal for enumeration of L. monocytogenes, but were inhibitory to other Listeria spp., 2) LPM agar was best overall in enumerating L. monocytogenes in foods, as it was the only medium to inhibit the growth of the 50 other organisms tested, while still supporting the growth of all L. monocytogenes strains. Cassiday and Brackett (1989) compared the enumeration of L. monocytogenes in artificially-inoculated hams and oysters using 10 direct plating media, including MMA, Dominguez medium, LEB agar, Despierres agar, LPM agar, and MVJ. The use of LPM agar gave the best recovery of the organism from ham, and Dominguez medium, from oysters.

No one medium has clearly emerged as superior. A direct plating medium efficient in recovering normal and injured L. monocytogenes from a variety of foods is needed to enable food microbiologists to detect and enumerate the organism in a wide range of foods.

Enrichment procedures. Cold enrichment was one of the earliest methods used to recover L. monocytogenes from food and environmental samples (Gray and Killinger, 1966). Samples were diluted in nutrient broth and stored at 4°C. After 24 h, and once a week thereafter, portions of the enrichment broth were plated onto selective media which were incubated at 35°C. With this procedure, detection of the organism can take 3 mo or longer. Incubation at 4°C suppresses the growth of most microorganisms, but Listeria spp. multiply slowly with a generation time of 1.5 d (Rosenow and Marth, 1987).

More recently, the incorporation of specific selective agents such as acridine dyes and nalidixic acid, into enrichment media has resulted in shortening the time required to isolate the organism (Klinger, 1988). Donnelly and Baigent (1986) modified Dominguez medium in developing

LEB, by adding nalidixic acid and acriflavin as selective agents. Doyle and Schoeni (1987) incubated foods in an enrichment broth containing acriflavine, polymyxin B and nalidixic acid as selective agents, under microaerophilic conditions of 5% O_2, 10% CO_2 and 85% N_2. One of the most commonly used selective enrichment procedures is that of Lovett et al. (1987) often referred to as the Food and Drug Administration (FDA) procedure. Samples are inoculated in an enrichment broth containing acriflavine, nalidixic acid and cycloheximide, and incubated at 30°C for 1 week. At 1 and 7 days, undiluted samples, as well as samples diluted in 0.5% KOH solution, are plated onto MMA for detection.

The highly selective LPM agar formed the basis of a detection procedure for use with meat and poultry (McClain and Lee, 1988). The primary enrichment broth, consisting of LEB modified to contain 20 (rather than 40) $mg.L^{-1}$ of nalidixic acid, was modified further to form the secondary enrichment broth containing 25 (rather than 12) $mg.L^{-1}$ of acriflavine. The primary broth, containing the sample, was incubated for 24 h at 30°C, after which 0.1 mL was transferred to 10 mL of secondary enrichment broth. This was incubated for 24 h at 30°C before plating on LPM agar for detection.

Several reports have been published on the comparison of enrichment procedures for the isolation of Listeria spp. from dairy products or meats. Doyle and Schoeni (1987) compared the detection of L. monocytogenes in cheese by: 1) cold enrichment in tryptose broth at 4°C over a period of 8 wk, 2) the FDA enrichment procedure, and 3) the Doyle and Schoeni enrichment procedure. L. monocytogenes was isolated from 41/90 (46%) samples of soft, surface-ripened cheese, with most isolations (21) being made from the cold enrichment procedure. In most cases, the organism was isolated from the cheese sample by only one of the three procedures. Slade and Collins-Thompson (1988) compared the isolation of Listeria spp. from milk by: a) the FDA enrichment procedure, and b) a procedure using a cold enrichment in tryptose broth, a selective enrichment in thiocyanate-nalidixic acid-acriflavine broth, followed by plating on McBride Listeria agar. L. innocua and L. monocytogenes were isolated from 19/34 (56%) of raw milk samples, with

both procedures being equally effective. Pini and Gilbert (1988) compared the detection of L. monocytogenes in chicken or soft cheese by a cold enrichment in tryptose phosphate broth at 4°C over a period of 12 wk, and the FDA enrichment procedure. L. monocytogenes was isolated from 70/160 (44%) chicken and from 23/222 (10%) cheese samples. Neither method alone yielded all isolates from the two food types.

1.3.2 Alternative Methods

In order to monitor the incidence of L. monocytogenes in foods, reliable methods must be developed for the rapid detection of the organism. Conventional methods are tedious, and variable in their results. Once repair and selective enrichment procedures have been optimized, methods for the rapid identification of food isolates need to be designed. Suggested techniques have included fluorescent antibody, enzyme-immuno assay, flow cytometry, and DNA hybridization (Klinger, 1988).

An unusual application of a physical detection method to food microbiology was reported by Donnelly and Baigent (1986) in their use of flow cytometry (FCM) for the detection of L. monocytogenes in raw milk samples. Fluorescently-labelled bacterial populations were passed rapidly through a laser beam and analyzed by FCM, allowing the characterization of a population of cells based on parameters such as morphology, DNA content and surface antigenicity. The DNA content was measured by its fluorescence intensity following propidium iodide staining. The FCM detection of the organism in raw milk was combined with a selective enrichment using LEM. FCM detected L. monocytogenes in artificially- and naturally-contaminated milks, with no interference from streptococci and staphylococci. A study comparing the microbial analysis of 939 raw milk samples by FCM following selective enrichment in LEB, and by a cold enrichment procedure, showed that of the 15 samples positive culturally for L. monocytogenes, FCM analysis showed a 5.9% false-positive and a 0.5% false-negative rate (Donnelly et al., 1988).

The production of monoclonal antibodies (MABs) for use in the identification of Listeria spp. in enzyme immunoassay (EIA) methods was reported by Farber and Speirs (1987b). Of the monoclones developed, 2 reacted only in the presence of flagellar antigen A, 3 with antigen B, and 1 with antigen C. These H antigens are found on L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri and L. seeligeri. There was no cross-reactivity with 30 non-Listeria cultures, including staphylococci and streptococci. One of the anti-B flagellar antigen MABs reacted with colony blots of L. monocytogenes on nitrocellulose (NC) membrane. When this reactivity was demonstrated by an enzyme-labelled goat anti-mouse IgA procedure, it allowed a presumptive identification of Listeria spp. in 2 days from naturally contaminated foods. The monoclones were also tested in preliminary experiments using colony blotting and microtitre plate EIA procedures on naturally-contaminated ground meats (Farber et al., 1988b).

The production of other MABs specific to the Listeria genus was reported by Butman et al. (1988). Tested by EIA and Western blots for specificity, the 15 IgG monoclones showed no cross-reactivity when screened against a panel of 21 other species, including Streptococcus spp. and Staphylococcus spp. The genus-specific antigen was identified as a heat-stable protein with a MW in the range of 30 to 38 kDa. Two of the MABs formed the basis of a commercial EIA method (Listeria-Tek) for detection of Listeria spp. in foods (Mattingly et al., 1988). McLauchlin and Pini (1989) have recently reported the use of MABs in the detection of L. monocytogenes in foods by a direct immunofluorescent test. Of 35 cheese samples, L. monocytogenes was detected by this direct test in 7 samples where the organism was present at $>10^3.g^{-1}$, and was not detected in 28 samples where the organism was either present at $<10^2.g^{-1}$, or was not isolated using conventional procedures (Pini and Gilbert, 1988). Another Listeria species-specific MAB was reported as detecting 10^6cells.mL^{-1} in enrichment broths after 24 h incubation by an ELISA procedure (Beumer and Brinkman, 1989).

The detection of L. monocytogenes by colony hybridization was first reported by Datta et al. (1987) using a radio-labelled DNA probe consisting of some 500 bp of the ß-hemolysin gene

(Flamm et al., 1989). The Listeria cells were irradiated by microwaves while in contact with the NaCl-NaOH lysing solution in order to break the gram-positive cell wall and denature the DNA. The method was tested against 52 pure cultures of Listeria spp. and homology was detected only with β-hemolytic (CAMP positive) strains. This DNA probe was used to detect L. monocytogenes in naturally-contaminated dairy products (Datta et al., 1988a). Although β-hemolytic L. monocytogenes represented only 1/100 to 1/1000 of the cells recovered from the food samples after direct plating, they were easily enumerated by colony hybridization. Isolates recovered from the plates after identification on the autoradiograms were CAMP positive. The 500-bp fragment was cloned into M13 bacteriophage vectors and sequenced by the dideoxynucleotide technique (Datta et al. 1988b). From this sequencing information, several oligodeoxynucleotides were synthesized and used as synthetic probes in a colony hybridization method to detect L. monocytogenes. The probes were specific when tested against 10 strains of the organism, and did not react with 9 strains of five other Listeria spp. nor with cultures of 13 other organisms. Two of the synthetic probes, AD07 and AD03, were tested with artificially-contaminated dairy products which were plated directly onto MMA and LPM agar. The LPM agar was more effective than the MMA in suppressing background flora. The number of spots on the autoradiograms of colony blots from LPM plates equalled 70-100% of the colonies that grew.

The development of specific probes based on unique regions of ribosomal RNA (rRNA) was exploited in a commercial hybridization assay for Listeria spp. in foods (Gene-Trak; Klinger and Johnson, 1988). The procedure is similar to the Gene-Trak Salmonella assay in that it involves membrane filtration of enrichment broth after incubation, followed by release of target DNA or RNA, and hybridization. In the Listeria assay, the target nucleic acids are genus-specific regions of 16S rRNA. Though the bulk of rRNA is highly conserved, small unique regions exist which form excellent targets due to their presence as multiple copies, thus increasing assay sensitivity. Sequence information on potentially variable regions in 16S rRNA was obtained, and oligodeoxynucleotides complementary to unique regions were synthesized as candidate probes.

These ^{32}P-labelled probes hybridized with all of the 139 Listeria strains tested, representing 16 serotypes, and against only one of the 72 other bacterial species. Though B. thermosphacta showed significant homology to the probes, it was negative in the final procedure due to its inability to grow at 35°C. When the detection of Listeria spp. in 151 food samples was compared using the FDA procedure and the hybridization assay, an agreement of 97.4% overall was obtained, with a 9.3% false-negative rate for the hybridization assay; there were no unconfirmed positive hybridization results. The company has recently announced the availability of this probe with a chromogenic label.

Four procedures to detect Listeria spp. in foods - the two commercially available alternative methods for detection of Listeria spp. (Listeria-Tek; Gene-Trak), the DNA probe for L. monocytogenes using LPM agar (FDA-LPM; Datta et al., 1987), and the FDA procedure - were compared on a total of 309 samples of milk and vegetables (Heisick et al., 1989) A sample was considered positive if Listeria were detected by at least one method, and if isolates were confirmed as Listeria spp. All 4 procedures yielded positive results with 58 or 59 of the 59 positive milk samples. With the 44 positive vegetable samples, the FDA-LPM method yielded 38 positive results (86%), with the other methods performing more poorly. The Listeria-Tek produced 22 false positive results, the others, none. The authors attributed the higher recovery of the FDA-LPM procedure partly to the superior performance of LPM agar.

Notermans et al. (1989) recently used a DNA probe to the L. monocytogenes delayed hypersensitivity factor to detect pathogenic serotypes of 284 strains of Listeria spp. Sequence homology to the probe was demonstrated for all 117 L. monocytogenes strains tested, except those of serogroup 4a, and for the L. ivanovii type strain. The hybridization reaction was negative for L. seeligeri, L. welshimeri, L. innocua, L. grayi and L. murrayi. The authors suggested that this probe would prove useful in the detection of L. monocytogenes in food and environmental samples.

Chenevert et al., (1989) have used the 650-bp Hind III internal restriction fragment from the listeriolysin O determinant of L. monocytogenes in Southern blots. Under stringent conditions, the probe reacted only with L. monocytogenes strains, and not with other Listeria spp.

1.3.3 Typing Systems

Serotyping of isolates is of little help in epidemiological studies because almost all strains of L. monocytogenes isolated from human cases belong to either serovars 1/2a, 1/2b or 4b. A phage-typing system was introduced consisting of a set of 28 phages isolated from lysogenic strains of Listeria (Audurier et al., 1984; McLauchlin et al., 1986). Recently, a new set of phages has been described (Loessner and Busse, 1990). Other suggested tests to use in epidemiological studies have been western blot analysis of surface proteins (Paquet et al., 1986), multilocus enzyme electrophoresis (Bibb et al., 1989; Selander et al., 1987; Piffaretti et al., 1989), DNA restriction analysis (Wesley, 1989), and ribotyping (Graves et al., 1991).

1.4 Hydrophobic Grid-Membrane Filters

Membrane filters (MFs) made of cellulose esters have been used since the 1940's in microbial analyses of water, beer, and sugar solutions (Moroz, 1957). Since 1963, MFs have also been made of other polymers such as: polyvinyl chloride, polysulfone, polycarbonate, polyester and nylon. These are manufactured by deposition from solvent and have pores with a twisted capillary structure. A 0.45μm pore size is commonly used in microbial analyses. MFs are now also available with straight pores produced by etching of nuclear particle tracks in polycarbonate (Nuclepore).

1.4.1 Uses in Food Microbiology

The advantages of MFs, compared to agar plates, in analytical food microbiology, include (Sharpe and Michaud, 1974; Sharpe and Peterkin, 1988):

1. Limits of detection are improved by filtration of larger volumes.
2. Foodborne organisms are not exposed to the risk of heat injury as in the preparation of pour plates.
3. Growth inhibitors and/or interfering substances are removed by the filtration procedure.
4. The MF can be transferred from one agar medium to another without loss of enumeration, e.g., from a resuscitation to a selective medium, or to a succesion of media for the determination of biochemical characterisitics.
5. Microbial identification reagents such as 4-diaminobenzaldehyde, enzyme-labelled antibodies, labelled nucleic acid probes, etc. can be applied directly to the organisms on the MF surface.

The hydrophobic grid-membrane filter (HGMF), consisting of a uniform grid of hydrophobic material printed on the surface of an MF polymer, was developed by Sharpe and co-workers (Sharpe and Michaud, 1974, 1975; Sharpe et al., 1978) as an experimental vehicle to provide sufficient growth compartments for a precise MPN count. It proved to have additional advantages and has been developed for commercial use in microbial analyses. The commercial product (Iso-Grid) is a 60 X 60 mm square of 0.45μm pore size polysulfone (Gelman Tuffryn), printed in a pattern of 40 X 40 grid cells using a black hydrophobic material.

The hydrophobic grid-cell walls limit the lateral growth of the microorganisms, so that square growths or "colonies" result. This microbial growth in an ordered array of 1600 compartments has the following additional advantages (Sharpe and Peterkin, 1988):

1. Increased numerical range and counting accuracy.
2. Increased counting precision.
3. Suitability for electronic counting due to the ordered array of growth compartments.
4. Suitability for accurate replication, also due to the known location of bacterial growth.

Treating the HGMF as a 1600-tube MPN device accomodates a concentration range of 4-log cycles in the original inoculum (Sharpe and Peterkin, 1988). The relationship between the HGMF count and the most probable number of growth-forming units (MPNGU) in the inoculum is given by:

$$MPNGU = N \log_e \frac{N}{N-X}$$

where N = the number of grid-cells (1600 for the Iso-Grid) and X = the number of grid-cells containing the growth of interest. The HGMF grid-cell is counted as one if it is positive for the organism of interest, no matter how many colonies it contains; it is counted as zero if it is negative.

Several methods of food microbial analyses using the HGMF have been reported (Lin et al., 1984; Entis and Boleszczuk, 1986; Holley and Millard, 1988; Peterkin and Sharpe, 1984; Peterkin et al., 1989a). Those which have received approval by the Health Protection Branch (HPB) and/or the AOAC include methods for total aerobic count, coliform count, faecal coliform count, E. coli count, including haemorrhagic colitis (HC) strains, V. parahaemolyticus count, P. aeruginosa count, and detection of Salmonella spp.

Many factors affected the filterability of foods through MFs, including pore size, anisotropy, adsorption and electrostatic effects (Sharpe et al., 1979). In order that MF-based methods be of practical value in analytical food microbiology, 1.0 mL of a 1:10 food suspension should filter in less than one minute. Sharpe et al. (1979) reported that 55 out of 58 foods tested filtered adequately without further treatment. Entis et al. (1982) stated that 69 out of 109 foods tested filtered well when prepared in a Stomacher with a diluent containing 1% Tween 80. For those foods which required additional treatment, enzyme digestion for 10 min improved filterability (Peterkin and Sharpe, 1980; Entis et al., 1982). Food suspensions for membrane filtration often require prefiltration to lessen the number of large food particles, which can have adverse effects on filtration rate and bacterial count (Sharpe and Peterkin, 1988). This can be provided by a

disposable pipette-tip prefilter with a 110 μm mesh which allowed full recoveries of E. coli and S. aureus cells from 8 food products, while providing the needed clarification (Peterkin and Sharpe, 1981).

An HGMF-based system of food microbial analysis with its potential for computerized detection, requires specific and sensitive chromogenic reagents such as enzyme-labelled antibodies and nucleic acid probes, for detection and identification. Peterkin and Sharpe (1984) showed that enterotoxigenic S. aureus can be detected using a horseradish peroxidase (HRP)-linked antibody to staphylococcal enterotoxin B (SEB) on a NCM blot taken from the growth on HGMF. The purple colour resulted from the action of the nascent oxygen from the HRP/H_2O_2 reaction, on the 4-chloro-1-naphthol oxygen acceptor (Hawkes et al., 1982). However, these blots could not be read automatically by the HGMF Interpreter, as the black border and grid-cell walls of an HGMF are needed by the computer in identifying the HGMF image (Sharpe and Peterkin, 1988). An improvement in this methodology enabling the colour to be developed directly on the HGMF was therefore required. An enzyme-linked antibody (ELA)-based method for the detection of Salmonella spp. directly on HGMFs was developed, using Spicer-Edwards flagellar antisera thus eliminating the NCM blotting step (Farber et al., 1985; Cerqueira-Campos et al., 1986). Pure cultures of 54 Salmonella spp. gave positive reactions; of 11 non-Salmonella organisms, only Citrobacter freundii was positive. A method using an HRP-conjugated MAB in an ELA procedure for the detection of E. coli O157 was developed which yielded presumptive identification from foods or enrichment broths, within 24h (Todd et al., 1988). Typing for the H antigen was performed on replicates taken from the HGMF before the ELA procedure. The method recovered E. coli O157 from a variety of artificially-inoculated meats, and from food samples obtained in two outbreaks of haemorrhagic colitis.

There are few reports of the use of HGMF in molecular genetics. Lo and Cameron (1986) used a simple immunological detection for screening a genomic library (GL) for a specific gene. The recombinant clones were filtered onto HGMF and, after incubation, were blotted to NCM.

These were developed with a primary rabbit antibody specific for the phenotypic product of the gene of interest. The presence of the product, and hence the gene, was demonstrated by the use of HRP-conjugated anti-rabbit antibodies. From these blots, the positively-reacting clones on the master HGMF were identified for further study.

1.4.2 Computerized Detection Methods

Though there have been many forms of the electronic colony counter to help the food microbiologist in counting agar plates, they have proved to be neither accurate nor popular. Sharpe (1978) examined the theoretical aspects of this problem through the use of Information Theory. Using the equation:

$$I = \log_2 P$$

where I = information measured in binary digits, and P = the set of alternatives, the author calculated that in counting the average plate with 30-300 colonies, the counter (whether electronic or human) need supply only 9 bits of information. However, the usual scanning electronic counter would supply 1,600,000 bits of information. This excess information creates a low signal to noise ratio, preventing the counter from providing an accurate count. In contrast, MPN methods supply 1 bit of information per growth compartment, so that there is a closer match between transmitting and receiving capacities (Sharpe and Peterkin, 1988). The major obstacle towards achieving the promised convenience and precision of the MPN method was the small number of growth compartments it was practical to inoculate individually. To increase the number of growth compartments, the first HGMFs were developed by dividing the surface of MFs using wax prints (Sharpe and Michaud, 1974, 1975; Sharpe et al., 1978). This development allowed a different approach to electronic counting (Sharpe et al., 1986; Sharpe and Peterkin, 1988). An HGMF counting system consisting of a colour or monochrome television camera with image monitor, and an IBM-XT (HGMF Interpreter), was self-calibrating, and able to give information as to the reliability of its data. Once the growth positions were determined, the system examined the reflectance data

from the grid-cells to determine which were occupied by the growth of interest. A comparison of automated counts by the HGMF Interpreter and manual counting by two analysts, of 7 types of food, showed no significant differences between the two counting methods at $\alpha = 0.05$ (Sharpe and Peterkin, 1988).

1.5 Purpose of Present Research

L. monocytogenes is a foodborne pathogen of growing importance to food manufacturers, regulatory agencies, and the consumer. It causes illness in pregnant women and their fetuses, the elderly, and immunocompromised persons, with a mortality rate of about 35%. It occurrs widely in the environment, and in foods, where it can survive and grow at refrigeration temperatures.

Present conventional methods for the detection, identification and enumeration of L. monocytogenes in foods are long and tedious. Due to the expense involved, food companies and regulatory agencies are unable to perform as many routine surveillance tests for the presence of the organism as is desirable for food safety; therefore, a more rapid, and preferably automated, detection method is required.

The system of analytical food microbiology based on the HGMF lends itself to the use of specific reagents which identify the organism of interest by colour cues. These reagents can be enzyme-labelled MABs or DNA probes, used with a substrate yielding an insoluble coloured product. Methods based on the use of these reagents can possess the required specificity and sensitivity, and can provide the coloured growth of the organism of interest needed by the HGMF Interpreter for automated detection and counting. The use of chromogenic, rather than radioisotope, labels on these reagents has the additional advantages of increased safety and lower costs.

The present research is undertaken to isolate DNA probes useful in the detection of L. monocytogenes in foods. As the hemolysin present in L. monocytogenes appears to be linked

to its virulence, a genotypic test for the determinant of this protein may form the basis of a specific identification. A genomic library of the organism is to be constructed and screened for clones expressing ß-hemolytic activity. The plasmid DNA from these recombinants, or their insert DNA, will be labelled and screened by colony hybridization against L. monocytogenes, other Listeria spp., and other potentially cross-reactive organisms and food pathogens, in order to identify a L. monocytogenes-specific DNA probe. The HGMF-based method using an enzyme-labelled probe will be tested for the detection and identification of L. monocytogenes in foods.

The L. monocytogenes genomic library will also be used to study virulence factors of this organism. The pathogenicity of the clones themselves is to be investigated. The insert DNA of the ß-hemolytic clones will be characterized by restriction mapping, and by probing against Southern blots of chromosomal and recombinant plasmid DNA.

Strains of L. monocytogenes of all serovars will be surveyed for native plasmids, and the molecular size of any plasmids found will be determined.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Strains of L. monocytogenes and other Listeria spp. were obtained from J.M. Farber and P. Ewan, HPB, Health and Welfare Canada, Ottawa, Ontario; B. Stewart, Animal Disease Research Institute, Agriculture Canada, Ottawa, Ontario; Laboratoire de Santé de la Province de Québec, Ste Anne-de-Bellevue, Québec; and American Type Culture Collection (ATCC), Rockville, Maryland, USA. Other organisms were from the HPB collection and from ATCC. Host cell strain E. coli C600 (F^-, thi-1, thr-1, leuB6, lacY1, tonA21, supE44) was obtained from J.-A. Dillon, HPB, Health and Welfare Canada, Ottawa, Ontario; and host cell strain E. coli DH5α (F^-, endA1, hsdR17(r_k^-,m_k^+), supE44, thi-1, λ^-, recA1, gyrA96, relA1, ϕ80dlacZ M15), from Gibco/BRL Canada, Burlington, Ontario. E. coli JM103 (pAD319), containing the cloned listeriolysin O gene, was obtained from A.R. Datta, FDA, Washington, DC, USA. Bacterial strains were maintained on Institut Pasteur Maintenance Medium at room temperature. As required, Listeria strains were streaked to tryptose agar (TA), and other organisms to brain-heart infusion (BHI) agar and incubated at 37°C, except for B. thermosphacta which was incubated at 30°C. Recombinant strains were maintained under selective pressure by use of the appropriate antibiotic.

2.2 Materials

Restriction endonucleases, T4-DNA ligase, bacterial alkaline phosphatase, Dnase I, DNA polymerase, low-melting (LM) agarose, pUC 18 and pBR322 vector DNA, DNA molecular size standards and Ham F-12 nutrient mixture were purchased from Gibco/BRL Canada, Burlington, Ontario; 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal), isopropyl-ß-D-thiogalactopyranoside (IPTG), ampicillin, tetracycline, proteinase K, calf intestinal alkaline phosphatase (CAP), deoxyribonucleotides and DNA Labeling and Detection Kit, Nonradioactive, from Boehringer Mannheim Canada (BMC), Dorval, Québec; lysozyme, cesium chloride (optical

grade), dimethylsulfoxide (DMSO), dithiothreitol (DTT), ethidium bromide (EtBr), ethylenediamine tetraacetate (EDTA), formamide, bovine serum albumin (BSA), polyethylene glycol (PEG) 8000, polyvinylpyrrolidone (PVP), sodium dodecyl sulfate (SDS), N-lauroyl sarcosine, Trizma buffer (Tris), and calf thymus DNA, from Sigma Chemical Co., St. Louis, MO, USA; Lugalvan G35 (polyethyleneimine) from BASF Canada, Toronto, Ontario; Gene-Clean from Bio 101 Inc., La Jolla, CA, USA; pZ523 spin columns from 5 Prime→3 Prime, West Chester, PA; Iso-Grid HGMF from QA Laboratories Ltd., Toronto, Ontario; Whatman 541 filter paper, from Thomas Scientific, Swedesboro, NJ, USA; agarose and nitrocellulose membrane, from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario; Schleicher & Schuell nylon 55 membrane, from Zymotech Inc., Montréal, Québec; ^{32}P-dATP, from ICN Biomedicals Canada, Ltd., Montréal, Québec; bacteriological media from Difco Laboratories, Detroit, Michigan, USA or Oxoid Canada Inc., Nepean, Ontario, and LiCl, potassium acetate and other chemicals, reagent grade, from BDH Inc., Toronto, Ontario.

The HGMF Interpreter and HGMF Replicator were developed and built in the workshop of the Automation Section, Bureau of Microbial Hazards, HPB. The sources of other equipment are indicated in the text.

2.3 Preparation of L. monocytogenes Chromosomal DNA

Chromosomal DNA for the genomic libraries was prepared from L.monocytogenes 81-861 (serovar 4b) by a modification of the method of Hadley and Deonier (1979). This strain was isolated from coleslaw implicated in the 1981 outbreak of listeriosis in the Maritime Provinces. Single colonies of the organism on TA were each inoculated into 4 tubes of BHI broth (5mL) and incubated for 18h at 37°C with shaking at 100 rev.min^{-1}. Each 5 mL of incubated broth was inoculated into 50 mL of BHI broth, and incubated as before. Cells were pelleted in sterile 50 mL capped centrifuge tubes in a Sorvall Model RC5C Centrifuge (Ingram and Bell Scientific, Don Mills, Ontario) at 10,000 X g for 15 min at 4°C in an SS-34 rotor. The pellet from 50 mL of culture was

washed with 10 mL of a 10 mM Tris-HCl (pH 8.0)-4mM EDTA solution, and pelleted as before. The resulting pellets were each gently mixed in 2.5 mL of a 50 mM Tris-HCl (pH 8.5)-5 mM EDTA-50 mM NaCl (TES)-10% sucrose solution until a smooth suspension was obtained. One mL of a freshly-prepared lysozyme solution (2.5 mg.mL^{-1}) was added to each cell suspension, and the mixture incubated for 60 min in a 35°C water bath. The cells were lysed by the addition of 50 mM Tris-HCl (pH 7.5)-62.5 mM EDTA-2.5 M LiCl-0.4% Triton X-100 (TELT; 2.5 mL for each pellet). Each tube was gently inverted 6 times and incubated for 60 min in a 35°C water bath. At this time the contents of the tube were clear and viscous. The DNA was sheared by gently pipetting the viscous liquid 15 to 20 times with a sterile wide-mouthed 10 mL pipette.

The crude DNA preparations were combined and placed in Beckman Quick-Seal tubes (16 X 76 mm; 12 mL volume), to which CsCl (0.6 g.mL^{-1}) and ethidium bromide (0.2 mg.mL^{-1}) were added. Sterile liquid paraffin was used to fill the tubes which were then heat-sealed. The sealed tubes were spun in a Beckman Model L8-M Ultracentrifuge (Beckman Instruments, Canada, Inc., Mississauga, Ontario) at 177,000 X g for 20 h at 4°C in a 70.1 Ti rotor The band of viscous, ethidium bromide-stained material (visualized under UV light) was collected by piercing the tube with a #19 syringe needle just below the band and allowing the DNA to drip into a sterile 5 mL tube. The DNA solution was extracted repeatedly with isopropanol saturated with 50 mM Tris-HCl (pH 8.0)-20 mM EDTA buffer (TE8) to remove the ethidium bromide, and dialyzed over a period of 2 days with 3 changes of 100 volumes each of TE8.

To recover the DNA by ethanol precipitation, the solution was made 0.3 M with respect to potassium acetate, and the DNA precipitated with 2 volumes of cold ethanol. After chilling at -20°C for 20 min, the DNA was pelleted at 16,000 X g in an Eppendorf microcentrifuge for 30 min at room temperature. The DNA pellet was washed in cold 70% ethanol, drained, dried for 10 min under vacuum at ambient temperature, and dissolved in 300 µL of TE8 (Dillon et al., 1985).

The DNA (75 µg in 300 µL) was further purified by incubation for 30 min at 37°C with 300 µL of a solution of 100 mM Tris-HCl (pH 8.0)-20 mM EDTA-5% sodium dodecyl sulfate containing

120 μg of proteinase K (final enzyme concentration = 400 $\mu g.mL^{-1}$). The incubation mixture was then extracted once with an equal volume of phenol/chloroform (1:1), and twice with an equal volume of chloroform/isoamyl alcohol (24:1), and the DNA recovered by ethanol precipitation as described above. The DNA concentration was determined from the 260 absorbance value, and its purity by a calculation of the 260/280 absorbance ratio, and by its appearance as a single band in an agarose gel after electrophoretic separation.

2.4 Preparation and Storage of Genomic Libraries

Of the genomic libraries prepared, the first (GL1) and the fourth (GL4), were used in the work described here. In the preparation of GL1, 10 μg of L. monocytogenes chromosomal DNA in a solution of 10 mM Tris-HCl (pH 7.5)-10 mM $MgCl_2$-75 mM NaCl-1 mM dithiothreitol (RB-high) was partially digested by incubation for 30 min at 37°C with Mbo I restriction endonuclease ($2U.\mu g^{-1}$ DNA). The fragments were separated by gel electrophoresis at $7V.cm^{-1}$ in 0.7% agarose with the fragments of a Hind III digest of lambda DNA as molecular size markers. DNA fragments in the 8-18 kb range were electroeluted into 2 mL of a solution of 40 mM Tris-HCl (pH 7.8)-20 mM sodium acetate-18 mM EDTA contained in a dialysis bag (Maniatis et al., 1982). The DNA, recovered by ethanol precipitation as described in Sect. 2.3, was dissolved in 10 μL of ligation buffer (20 mM Tris-HCl (pH 7.6)-10 mM $MgCl_2$-10 mM dithiothreitol-0.6 mM ATP). Plasmid vector pBR322 DNA (1 μg) in RB-high was completely cut by incubation for 30 min at 37°C with BamH I restriction endonuclease ($8U.\mu g^{-1}$ DNA). The cut vector DNA was then purified by extraction with phenol/chloroform (1:1) followed by chloroform/isoamyl alcohol (24:1) (Maniatis et al., 1982), precipitated with ethanol as previously described, and finally dissolved in ligation buffer (10 μL). BamH I-cut pBR322 DNA (10 μL; ≈1 μg) and L.monocytogenes size-separated DNA fragments (10 μL; ≈2 μg) were mixed, 0.2 U of T4 DNA ligase were added, and the ligation mixture incubated for 16-18 h at 14°C (Dillon et al., 1985). Competent E.coli C600 cells were then prepared and transformed by the method of Dillon et al. (1985). Cells were inoculated into 50 mL

of 1% tryptone-0.5% yeast extract-0.05% NaCl broth (L broth), and grown to mid-log phase by incubation for 2 h at 37°C with rotary shaking at 120 rev.min^{-1}. After chilling on ice for 15 min, the cells were pelleted by centrifugation at 7000 X g for 10 min at 4°C. The cells were resuspended and pelleted successively in 1 volume of cold 0.1 M $MgCl_2$, and 0.5 volumes of cold 0.1 M $CaCl_2$, and finally resuspended in 0.05 volumes of cold 0.1 M $CaCl_2$ before incubating at 4°C for 6 h. Chilled ligation mixture (20 µL; 3 µg DNA) was added to competent cells (200µL), the mixture was chilled on ice for 30 min, heated in a 42°C water-bath for 2 min, then chilled again for 10 min. The mixture was added to sterile L broth (3 mL), which had been tempered to 37°C, and incubated for 2 h at 37°C with gentle shaking. Fifty µL each of transformed cell dilutions (10^0, 10^{-2}, 10^{-4}) were spread-plated on tryptic soy agar containing ampicillin at 50 µg.mL^{-1} (TSA Ap_{50}), and incubated overnight at 37°C. After determination of the transformation rate, Iso-Grid HGMFs were inoculated by filtration with the transformed cell dilutions, at a level calculated to deposit 60 transformants per filter, and incubated on TSA Ap_{50} as before (Peterkin et al, 1987). Transformants with insertional inactivation were identified by inoculating copies using the HGMF Replicator (Fig. 2-1; Automation Section, Bureau of Microbial Hazards, HPB), and incubating the replicates overnight at 37°C on TSA plates containing tetracycline at 15 µg.mL^{-1} (TSA Tet_{15}).

In the preparation of GL4, 10.5 µg L.monocytogenes chromosomal DNA in 25 µL of a solution of 50 mM Tris-HCl (pH 8.0)-10 mM $MgCl_2$-50 mM NaCl was partially digested by incubation for 15 min at 37°C with Mbo I restriction endonuclease (0.25 U.µg^{-1} DNA) Fragments in the 8-12 kb range were separated by electrophoresis on a 0.7% LM agarose gel, and purified by adsorption to and elution from small glass beads (Glasmilk; Gene-Clean kit), according to the manufacturer's instructions. Plasmid vector pUC18 DNA (0.5 µg in 2 µL of RB-high) was completely cut by incubation for 2 h at 37°C with BamH I restriction endonuclease (20 U.µg^{-1} DNA). After checking for complete digestion by agarose gel electrophoresis, the linearized vector DNA was recovered by ethanol precipitation as previously described (Sect. 2.3), and dissolved in 10 µL of TE8 (50 ng.µL^{-1} DNA). Linearized vector DNA (0.5 µg) was dephosphorylated with

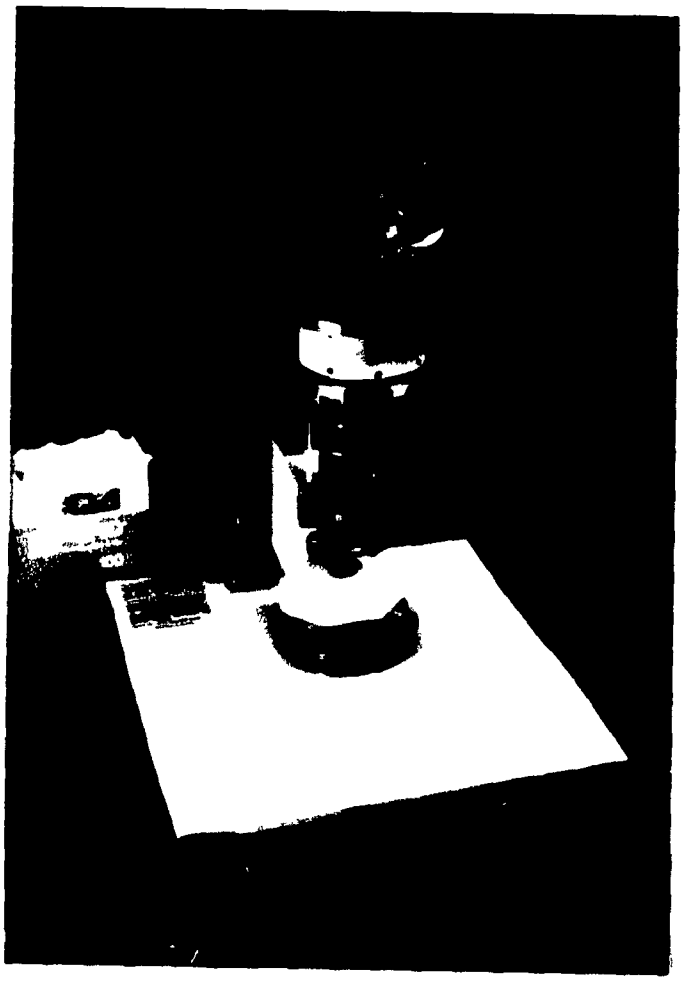

Fig. 2-1. HGMF Replicator. An HGMF with the border "T" at the bottom is aligned under the viewer cross hairs. The head is actuated so that the inoculator points pick up growth from the corresponding HGMF grid cells. A sterile HGMF is inoculated in the same pattern by repeating the operation. The inoculator, which may contain 1600, 400, or 100 points, is removed and sterilized after six copies.

CAP (40 U.ng^{-1} DNA) in 0.5 M Tris-HCl (pH 9.0)-10 mM $MgCl_2$-1 mM $ZnCl_2$-10 mM spermidine buffer (Maniatis et al., 1982), recovered by ethanol precipitation and dissolved in 10 µL of TE8 (50 ng of DNA/µL). The L. monocytogenes DNA fragments (2.5 µL; ≈250 ng) and BamH I-cut pUC18 DNA (1 µL; ≈50 ng) were mixed, at an insert/vector molar ratio of 2/1 (Maniatis et al., 1982), in 50 mM Tris-HCl (pH 7.6)-10 mM $MgCl_2$-5% PEG 8000-1 mM ATP-1 mM DTT buffer and incubated for 42 h at 5°C, at which point T4 DNA ligase (0.1 U.µg^{-1} DNA) was added and the incubation continued for a further 24 h. Dephosphorylated linearized vector DNA (1 µL; ≈50 ng) was also used, at an insert/vector molar ratio of 1/1, under the same conditions. Competent E coli DH5α cells were prepared and transformed by the procedure of Hanahan (1983). Cells which had been grown to mid-log phase in 2% tryptone-0.5% yeast extract-10 mM NaCl-2.5 mM KCl-10 mM $MgCl_2$-10 mM $MgSO_4$ (SOB medium) at 37°C for 2 h with rotary shaking at 275 rev min^{-1}, were chilled on ice for 10 min and pelleted at 1000 X g for 10 min at 4°C. The cells were resuspended and pelleted successively in 0.3 volume of 10 mM MES (pH 6.2)-100 mM RbCl-45 mM $MnCl_2$-10 $CaCl_2$-3 mM hexamine $CoCl_3$ (TFB), and 0.08 volume of TFB. With 5 min on ice after each step, DMSO was added to 3.5%, DTT was added to 75 mM, and additional DMSO was added to a final concentration of 7% in the competent cell suspension. These cells were stored at -70°C for up to 6 mo, without loss of transformation efficiency. One µL of chilled ligation mixture, diluted 1:5 in TE8 and containing 3 ng of DNA, was added to 100 µL of competent cells in 17 X 100 mm polypropylene tubes (Falcon 2059). The mixture was chilled on ice for 30 min, heated in a 42°C water-bath for 45 s, then chilled on ice for 2 min. SOB medium with 20 mM glucose (SOC medium; 900 µL), was added to the cell mixture which was incubated for 1 h at 37°C with shaking at 225 rev.min^{-1}. One hundred µL each of transformed cell dilutions (10^0, 10^{-1}) were gently spread-plated on 1% tryptone-0.5% yeast extract-0.5% NaCl-1.5% agar containing ampicillin (50µg.mL^{-1}), X-Gal (0.004%), and IPTG (0.1 mM) (LB_{AX} agar), and incubated overnight at 37°C. Colonies of transformed cells were blue; colonies of cells containing recombinant plasmids were colourless.

Storage. The genomic library GL4 was stored in the following ways:

1. A gene bank preparation was obtained by adding 9 X 10^3 cells of the culture of E. coli DH5α which had been transformed with the ligation mixture, to 15 mL of BHI broth containing ampicillin (50μg.mL^{-1}; BHI Ap_{50}). After overnight growth at 37°C, with shaking at 225 rev.min^{-1}, aliquots (1 mL) of the culture were mixed with 0.25 mL of sterile glycerol in sterile 2 mL tubes which were screw-capped and stored at -70°C.

2. Each of the 2500 clones screened on 7% horse blood agar plates for expression of hemolytic activity, were stored as follows: 0.5 mL of an overnight culture of the clone in BHI Ap_{50} was mixed with 0.5 mL of sterile 40% glycerol in sterile 2 mL tubes, which were screw-capped and stored at -70°C.

3. The same 2500 clones were deployed and replicated (see Sect. 2.11) on sets of seven HGMFs (400 clones per filter) placed on BHI agar plates containing 20% glycerol. The replicated HGMFs were incubated at 35°C for 6 h, and stored at -70°C. The viability of the clones was checked every month for the first six months of storage, and each six months afterwards, by allowing the plates to thaw at room temperature for 2 h, transferring the HGMF to fresh BHI agar, and incubating overnight at 35°C before checking for growth of all the strains (Peterkin et al., 1987; Sharpe et al., 1989).

2.5 Preparation of Plasmid and Insert DNA

Plasmid DNA was prepared on a large-scale by a modification of the procedure of Ng et al. (1987). Single colonies of recombinant clones were inoculated to 6 TSA plates containing ampicillin (100 μg.mL^{-1}), and incubated overnight at 37°C. The cells were harvested, suspended in 25 mM Tris-HCl (pH 8.0)-2 mM EDTA-0.2% glucose (4 mL) in a sterile 50 mL Sorvall tube, and

thoroughly mixed before adding lysozyme (1mL; 10 mg). The suspension was held on ice for 20 min, and the cells lysed by adding 10 mL of a 0.2 N NaOH-1% SDS solution. After 5 min on ice, 7.5 mL of 3 M sodium acetate, pH 4.8, was added. The tube containing the lysate was held on ice for 30 min, during which time it was inverted gently several times, and then centrifuged in a Sorvall Model RC5C Centrifuge (Ingram and Bell Scientific, Don Mills, Ontario) at 18,700 X g for 15 min at 4°C. The supernatant was recovered without disturbing the pellet.

2.5.1 CsCl Density Gradient Centrifugation

After dividing the supernatant between 2-50 mL Sorvall tubes, the DNA was recovered by precipitation with ethanol as previously described (Sect. 2.3). Each DNA pellet was dissolved in 7 mL of TE8 and added to each of 2 conical 10 mL tubes containing 7 g of CsCl. The tubes were capped and their contents mixed by inversion until the CsCl dissolved. Two hundred µL of EtBr (2 mg) was added, and the volume was brought to 10 mL with TE8. After the tube contents were mixed by inversion, they were transferred to Beckman Quick-Seal tubes (16X76 mm; 12 mL volume). These were filled up with sterile liquid paraffin, heat-sealed and centrifuged in a Beckman Model L8-M Ultracentrifuge (Beckman Instruments, Canada, Inc., Mississauga, Ontario) at 207,000 X g for 21 h at 10°C in a 70.1 Ti rotor. When the tube was visualized under UV light, the plasmid DNA was seen as the lower band stained by EtBr. It was collected by piercing the tube just below the band with a #19 needle, bevel side up, attached to a 1 mL syringe which was used to remove the DNA. The DNA solution after collection was extracted repeatedly with TE8-saturated isobutanol to remove the EtBr, and then transferred to a 50 mL Sorvall tube. After sterile distilled water (2 volumes) was added, the DNA was precipitated with ethanol as previously described (Sect. 2.3), and pelleted in a Sorvall centrifuge at 18,700 X g for 30 min at 4°C in an SS-34 rotor. The pellet, after washing with 70% ethanol, was dissolved in 100 µL of TE8. The concentration and purity of the DNA were determined as previously described.

2.5.3 pZ523 Spin Column

In later large-scale preparations of plasmid DNA, the CsCl density gradient separation step of the above procedure was replaced by the spin column procedure of Felsenstein (1988). Cell growth and lysis were performed as described in Sect. 2.5.

NaCl (5M; 0.1 volumes) was added to the supernatant in a 50 mL Sorvall tube and mixed gently. DNA was precipitated by PEG 8000 (10% w/v) which was added as a solid, and left to dissolve for 4 h to overnight at 4°C. The DNA was pelleted by spinning at 3,000 X g for 5 min at 4°C, dissolved in 2 mL of a solution of 10 mM Tris-HCl (pH 8.0)-1 mM EDTA-1 M NaCl (Solution D), and, finally, extracted 3 times with equal volumes of chloroform:isoamyl alcohol (24:1). The DNA was recovered by precipitation with cold ethanol as previously described (Sect. 2.3), dissolved in 1.8 mL of Solution D, and purified on a pZ523 spin column according to the manufacturer's directions. The DNA pellet recovered after this procedure was dissolved in 2 mL of TE8, and purified from residual contaminating RNA by digestion at room temperature for 1 h with 100 µg of DNase-free RNase. After ethanol precipitation as previously described (Sect. 2.3), the pellet was dissolved in a suitable amount of TE8, and the concentration and purity of the DNA were determined as previously described (Sect. 2.3).

2.5.3 Plasmid Mini-Preparations

Strains of L. monocytogenes and other Listeria species were examined for the presence of plasmids using the screening procedure of Anderson and McKay (1983), modified by the addition of an RNase digestion following lysis. Overnight cultures (1 mL) of L. monocytogenes in BHI broth in microtubes were spun at 16,000 X g in an Eppendorf microcentrifuge for 10 min, the cell pellet resuspended in 380 µL of 50 mM Tris-HCl (pH 8.0)-1 mM EDTA-6.7% sucrose, and 100 µL of lysozyme solution (1mg) were added. After a 10 min incubation at 37°C, TE8 buffer (50 µL) and 20% SDS (25 µL) were added, the incubation was continued for 10 min, and 3 N NaOH (25 µL) was added. The freshly-lysed cells were mixed gently for 10 min, and then neutralized with

2 M Tris-HCl, pH 7.0 (50 µL). The preparation was purified from residual contaminating RNA by digestion on ice for 10 min with DNase-free RNase (2µg.mL^{-1}; 1.5 µL). This was followed by a phenol-chloroform extraction and ethanol precipitation as previously described (Sect. 2.3) to recover the purified plasmid DNA.

Small scale preparations of the recombinant plasmids were performed by the LiCl boiling method of Wilimzig (1985). Overnight cultures of recombinant clones in BHI broth (1 mL) were spun at 16,000 X g in an Eppendorf microcentrifuge for 5 min, the cell pellet was completely resuspended in 100 µL of a solution of 50 mM Tris-HCl (pH 7.5)-62.5 mM EDTA-2.5 M LiCl-0.4% Triton X-100 (TELT), and 10 µL of lysozyme solution (100 µg) were added. The tube was capped and placed in a boiling water bath, or a 98 °C heating block, for 1 min, chilled on ice for 5 min, and centrifuged at 16,000 X g at room temperature for 10 min. The supernatant (100 µL) was transferred to a fresh tube, and the DNA recovered by precipitation with ethanol as previously described (Sect. 2.3). The pellet, after washing in 70% ethanol, was resuspended in a volume of TE8 buffer appropriate for the use to which the DNA was to be applied.

2.5.4 Preparation of Insert DNA

Plasmid DNA from each of the ß-hemolytic clones was prepared by either the CsCl density gradient or the pZ523 column procedures, as previously described. The insert DNA was cut from each of these plasmids by successive digestions with Kpn I and Pst I. Fragments from these preparative scale double digests were separated by horizontal agarose (0.7%) gel electrophoresis at 7 V.cm^{-1} of gel, in a Sub Cell (Bio-Rad Laboratories, Canada, Ltd., Mississauga, Ontario), using 40 mM Tris-HCl (pH 7.8)-20 mM sodium acetate-1.8 mM EDTA electrophoresis buffer (TAE). Molecular size markers consisted of fragments of a Hind III digest of lambda phage DNA. Insert DNA was obtained by electroelution of the desired fragment into troughs filled with LM agarose, removal of the LM agarose pieces, and recovery of the DNA from the LM agarose using the Gene-Clean kit, according to the manufacturer's directions.

2.6 Plasmid Molecular Size Determination and Restriction Mapping

Plasmid DNA from L. monocytogenes strains prepared on a small scale by the procedure of Anderson and McKay (1985), or recombinant plasmid DNA from ß-hemolytic clones, prepared by the LiCl-boiling method or by the pZ523 column procedure, was separated by horizontal gel electrophoresis in 0.7% agarose at 7 $V.cm^{-1}$ of gel, in a Sub Cell (Bio-Rad Laboratories, Canada, Ltd., Mississauga, Ontario), using 40 mM Tris-HCl (pH 7.8)-20 mM sodium acetate-1.8 mM EDTA electrophoresis buffer (TAE). The migration distance of the covalently-closed circular (CCC) form of the plasmid DNA was compared with that of a set of molecular size markers, consisting of supercoiled DNA of known molecular sizes from 2.07 to 16.21 kb. The migration distance of each marker was plotted on semi-log paper against its molecular size; the migration distance of the plasmid DNA was measured, and its molecular size extrapolated from the plot. The molecular size of pUC18 vector, 2686 bp, was subtracted from the value for each recombinant plasmid to obtain the insert size.

A series of single restriction digests with BamH I, EcoR V, HincIII, Hind III, Kpn I, Pst I, and Pvu II, under reaction conditions appropriate for each enzyme, were performed on plasmid DNA from ß-hemolytic clones, prepared by the LiCl-boiling method or by the pZ523 column procedure. The DNA fragments were separated by gel electrophoresis in a horizontal 0.7% or 1.5% agarose gel in TAE at 7 $V.cm^{-1}$, and their migration distances compared to those of a set of molecular size markers of linear DNA, to obtain the molecular sizes of the plasmid restriction fragments. The markers were either a Hind III digest of lambda phage DNA, consisting of 7 fragments with molecular sizes ranging between 23.6 and 0.56 kb, or a ladder of multiples of a 123 base pair fragment for accurately determining the molecular sizes of fragments less than 1 kb in size. A provisional map of restriction sites in the inserts was deduced from this information, sizes of fragments from Hind III/Pvu II double digests were deduced, and confirmed by experiment.

2.7 Determination of ß-Hemolytic (Cytolytic) Activity

Blood agar plates. Clones (2500) of GL4 were screened for ß-hemolytic activity by stabbing a toothpick inoculated with cells of the clone, into plates of 7% horse blood agar. Controls of ß-hemolytic (L. monocytogenes 81-861) and non-hemolytic (L. innocua LA-1) Listeria spp. were inoculated on each plate in the same way. After overnight incubation at 37°C, the plates were examined by transmitted light for zones of clearing, demonstrating the presence of ß-hemolysis. Recombinant plasmid DNA, isolated by the pZ523 column procedure (Sect. 2.5.3) from clones showing ß-hemolytic activity, was used to transform competent E. coli DH5α cells, and the ß-hemolytic activity of the transformants was determined in the same way.

Preparation of broken cell fractions. To prepare sonicated cell extracts, overnight cultures (5 mL) in BHI broth incubated at 35°C were transferred to 15 mL conical centrifuge tubes and sonicated for 110 s on ice, using the Branson Sonifier 250 with a precooled microtip, set at 50% output and 50% duty cycle. Microscopic examination was used to confirm cell disruption A portion (3 mL) of the sonicated extract was centrifuged at 1000 X g for 25 min, and the pellet resuspended in PBS buffer (3 mL). The sonicated extract, the resuspended pellet and its supernatant, each with and without 6 mM cysteine, were tested for hemolytic activity.

To prepare lysates, the method of Leimeister-Wachter and Chakraborty (1989) was followed. BHI broth (10 mL) was inoculated with starter culture (1 mL) in BHI broth, and incubated overnight at 30°C (clones), or 35°C (Listeria spp). The cells were pelleted and resuspended in 0.1 volumes of PBS (10mM, pH 5.5) and lysozyme (10 $mg.mL^{-1}$) was added. The suspension was incubated at ambient temperature for 30 min and then subjected to three cycles of freezing and thawing. After centrifugation at 10,000 X g for 10 min at 4°C, the supernatant, with and without 6mM cysteine, was tested for hemolytic activity.

Hemolysin titre. The organisms tested were ß-hemolytic clones, and controls of L. monocytogenes 81-861, L. innocua LA-1, E. coli DH5α, E. coli DH5α(pUC18) and a non-hemolytic clone designated #1-2. Supernatants of cultures grown overnight at 37°C in 2%

tryptose-0.2% glucose-0.5% NaCl-0.25% Na_2HPO_4, pH 7.3 (TPB), or, alternatively, broken cell fractions, were assayed for hemolytic activity in 96-well, U-bottomed microtitre plates. Portions of each test preparation were made 6 mM with respect to cysteine. A suspension (2%) of washed sheep red blood cells was prepared in 0.01 M phosphate buffered saline, pH 7.2 (PBS). Doubling dilutions of the preparation to be tested were made in PBS in the plate wells, and the sheep red blood cells were added in a 1:1 ratio (50 µL of each). The plates were incubated at 37°C overnight, and examined for hemolysis of the red blood cells at 2 h and 24 h. A positive result was indicated by a complete disappearance of the red blood cells. The titre was the reciprocal of the last dilution showing complete hemolysis.

CAMP test. Standard strains of S. aureus, Rhodococcus equi, controls of L. monocytogenes 81-861, L. innocua LA-1, L. ivanovii LA-2, test organisms consisting of the ß-hemolytic clones, E. coli DH5α, E. coli DH5α(pUC18) and the non-hemolytic clone #1-2, were streaked on TSA plates and incubated overnight at 37°C. Single colonies of S. aureus and of R. equi were streaked down each edge of a TSA plate containing 5% sheep red blood cells. Single colonies of the test and control organisms were streaked across the plate such that the inoculum approached, but did not touch, the streaks of S. aureus and R. equi. Duplicate plates were incubated 24 h at 37°C, or 48 h at 25°C in an atmosphere of increased (10%) CO_2. A positive CAMP test for L. monocytogenes was indicated by a zone of hemolysis enhanced by the S. aureus growth; the zone of hemolysis of L. ivanovii was enhanced by R. equi.

Cytolysin titre. Test organisms consisting of the ß-hemolytic clones, E. coli DH5α, E. coli DH5α(pUC18) and the non-hemolytic clone #1-2 , and controls of L. monocytogenes 81-861, and L. innocua LA-1, were grown overnight at 35°C with shaking in 10 mL of TPB. They were pelleted at 10,240 X g in an IEC Centra 8 bench-top centrifuge (Fisher Scientific, Ottawa, Ontario) for 25 min, and the supernatants were each sterilized by membrane filtration through a syringe-tip filter unit, 0.22 µm pore size (Millex-GS; Millipore Ltd., Mississauga, Ontario). Portions of the sterile fractions were made 6 mM with respect to cysteine. Chinese hamster ovary (CHO) cells were

grown as monolayers in Ham F-12 nutrient medium supplemented with 10% fetal bovine serum. For assay, freshly-seeded cells were treated with 0.5% trypsin in PBS (0.7 mL) at 37°C for 5 min, and then suspended in F-12 nutrient medium with 1.0% serum, at a final cell density of 2 X 10^4.mL^{-1}. The cell suspension (100 µL) was dispensed to microtitre plate wells, and undiluted sterile test and control fractions (10 µL) were added. The plate was incubated for 24 h at 37°C in 5% CO_2, and then examined microscopically for evidence of cell lysis. A positive test was indicated by the appearance of granular, ruptured CHO cells. The cytolysin titre was the reciprocal of the highest dilution of supernatant showing a positive test.

2.8 Mouse Pathogenicity Test

The ß-hemolytic clones to be tested, and the controls of L. monocytogenes 81-861, L. innocua LA-1, E. coli DH5α, E. coli DH5α(pUC18) and the non-hemolytic clones #1-2 and #10-50, were grown on BHI agar overnight at 37°C. At the same time, the hemolytic activity of the test clones was checked on blood agar plates as previously described (Sect. 2.7). Starter cultures were prepared by inoculating a single colony from each BHI agar plate into tryptic soy broth containing 0.6% yeast extract (TSBYE) and incubating as before. TSBYE (10 mL) was inoculated with 1 drop of starter culture and incubated as before. The cells were pelleted in a Sorvall RC5C Centrifuge (Ingram and Bell Scientific, Don Mills, Ontario) at 10,000 X g for 15 min at 4°C in the SS-34 rotor. and resuspended in 1 mL of a 0.1% peptone solution. Five female mice (Charles River CD-1; 16-18 g) were each injected intra-peritoneally with 0.1 mL of cell suspension (10^9 cells) for each of the 13 organisms tested. The mice were given food and water ad lib., and observed for death over a period of 7 days.

2.9 DNA Labelling

Plasmid or insert DNA from each of the ß-hemolytic clones, or from recombinant plasmid pAD319, was prepared as previously described. The DNAs were labelled either by nick-translation

with a radioisotope, or, for chromogen labelling, by direct cross-linking with an enzyme, or random-primed labelling with a hapten.

2.9.1 Radiolabelling

Plasmid or insert DNA was labelled by nick-translation with E. coli DNA polymerase I using ^{32}P-labelled dATP according to established procedures (Maniatis et al., 1982). The DNA (1 μg) was added to a reaction mixture containing 500 mM Tris-HCl (pH 7.2), 100 mM $MgSO_4$, BSA (2.5 μg), dGTP, dCTP, dTTP (1nmole each), α-^{32}P-dATP (200 pmol; 100 μCi), DTT (1 nmol), DNase I (0.05 ng), and DNA polymerase I (5 U). The mixture was incubated at 37°C for 2 h and the labelled DNA was recovered by precipitation with ethanol as previously described (Sect. 2.3). Just before use the probe was denatured by heat (5 min;100°C) and chilled on ice.

2.9.2 Chromogen Labelling

Direct labelling. Plasmid or insert DNA was labelled with either HRP or AP by a modification of the Renz and Kurz method (1984). The enzyme was first conjugated to PEI, a polycation (MW = 1500 Da), by the following procedure. Twenty mg of HRP (5000 U) were dissolved in 220 μL of 50 mM sodium phosphate buffer (pH 6.0), a 60 μL aliquot of a p-benzoquinone solution (30 mg.mL^{-1} of ethanol) was added, and the reaction mixture was left for 1 h in the dark at ambient temperature. Activated HRP was separated from nonreacted benzoquinone by Sephadex G100 column chromatography with NaCl (150 mM) as the running solution. Brown fractions of activated HRP were combined (about 2 mL) and the pH of the combined fractions was raised by the addition of 0.1 vol of 1 M sodium bicarbonate. PEI (Lugalvan G35 - 133 μg; 2.7 μL) was added, and the reaction mixture was incubated overnight in the dark at 37°C while cross-linking of HRP and PEI occurred. The HRP-PEI conjugate was concentrated 5-fold by dialysis against 20% PEG 8000 at 4°C for 3 h. The enzyme activity was

retained for at least 6 mo when stored at 4°C. AP (3 mg; 6900 U) was conjugated to PEI in the same way.

Before labelling, plasmid DNA was linearized by incubation for 2 h at 37°C with Kpn I restriction endonuclease (10 $U.\mu g^{-1}$ DNA). Either plasmid or insert DNA (1μg) was dissolved in 10 μL of 5 mM sodium phosphate buffer (pH 6.8), and denatured by heating for 5 min at 100°C, followed by 5 min chilling on ice. HRP-PEI conjugate (2 μL), 5% glutaraldehyde (6μL), and sodium phosphate buffer (5 mM, pH 6.8; 18 μL) were added and gently mixed. The sample was incubated for 10 min at 37°C, and added directly to the hybridization solution (see Sect. 2.10).

Random primed labelling. A hapten, digoxigenin (Dig), was incorporated into the DNA by random primed labelling, using the DNA Labeling and Detection Kit, Nonradioactive (Boehringer Mannheim, 1988). Linearized plasmid, or insert, DNA (1 μg) was denatured by heat (5 min; 100°C) and chilled on ice. Hexanucleotide primer, dATP, dCTP, dGTP, Dig-dUTP, DTT, and Klenow fragment of Pol I (2 U) in a pH 6.5 buffer were added, and the reaction was allowed to proceed for 2 h at 37°C. The DNA was recovered by precipitation with cold ethanol according to the procedure previously described (Sect. 2.3), and dissolved in TE8 buffer (50 μL). As required, labelled DNA (12.5 μL; 250 ng) was denatured by heat (5 min; 100°C) and added directly to the hybridization solution.

2.10 DNA/DNA Hybridization

Radiolabelled DNA probes were used both for hybridization with colony blots on cellulose filter paper, and for direct colony hybridization on HGMFs. Chromogen-labelled probes were used both for direct colony hybridization on HGMFs, and for hybridization with Southern blots. To lyse Gram positive as well as Gram negative organisms, it was necessary to include a high temperature heating step whilst the membranes holding the colonies were in contact with the alkaline lysis-denaturation solution (Datta et al., 1987).

2.10.1 Southern Blots

As required, plasmid DNA from the hemolytic recombinant clones was sequentially digested with Kpn I and Hind III restriction endonucleases. The DNA fragments were separated by horizontal gel electrophoresis in a 15 cm 0.8% agarose gel in Tris-acetate buffer (pH 8.0), run for 6 h at 60 V (4 $V.cm^{-1}$). The DNA fragments were transferred to a nylon membrane (Nytran; Schleicher & Schuell, Keene, NH, USA) in a vacuum blotting apparatus (Vacugene; Pharmacia Canada Ltd., Dorval, Québec), run at 40 mm Hg. The DNA was first depurinated by treatment for 10 min with 0.25 N HCl, followed by denaturation and transfer by treatment with 0.4 N NaOH (Aguinaga et al., 1989). The denatured DNA was fixed to the surface of the nylon membrane by UV irradiation of 254 nm at a 10 cm distance for 5 min [100 $ergs.(mm^2)^{-1}.s^{-1}$]. The instructions of the DNA Labeling and Detection Kit, Nonradioactive (Boehringer Mannheim, 1988) were followed for the hybridization of the membrane-bound DNA to a Dig-labelled DNA probe (see Sect. 2.9.2). The probes used were Kpn I - cut pIP1, pIP2, pIP5, pIP7, or pAD319, the latter containing the cloned listeriolysin O gene. Pre-hybridization was carried out in a plastic bag containing a solution of 5 X standard saline citrate-0.5% gelatine-0.1% sodium N-lauroyl sulphate-0.02% SDS (hybridization solution). Standard saline citrate (SSC) is a solution of 0.15 M NaCl-0.015 M sodium citrate, pH 7.0. After the bag was sealed, it was incubated at 68°C for 2 h with gentle shaking. The solution was replaced with hybridization solution containing freshly-denatured Dig-labelled DNA (≈250 ng), the bag re-sealed, and incubation continued at 68°C for 6-16h, with gentle shaking. The labelled DNA probe was recovered by pouring into a sterile 15 mL conical centrifuge tube, which was screw-capped and stored at -20°C. The membrane was transferred to a lidded plastic box, washed twice for 5 min at ambient temperature with 2 X SSC-0.1% SDS, and twice for 15 min at 68°C with 0.1 X SSC-0.1% SDS. The presence of the Dig-labelled probe was detected using an anti-Dig antibody, according to the procedure of the manufacturer (Boehringer Mannheim, 1988). The membrane was incubated at ambient temperature for 1 h in a solution of AP-labelled anti-Dig antibody, washed, and placed in a substrate solution of NBT/BCIP in a sealed

NBT/BCIP in a sealed bag in the dark at ambient temperature for up to 16h. The enzyme-substrate reaction, yielding a dark blue precipitate at the site of hybridization, was stopped by washing the membrane in TE8.

2.10.2 Colony Blots on Cellulose Filters

A Whatman #541 cellulose filter (85 mm diam.) was applied to the surface of an agar plate containing colonies, with the aid of a bent glass rod, and left for 5 min. This filter was transferred, colony side up, to a filter paper which had been soaked with 2 mL of a 0.5 N NaOH 1.5 M NaCl solution (lysis-denaturation solution), and exposed to microwave irradiation (≈ 700 watts) for 30 s. The cellulose filter was then transferred to a filter paper which had been soaked with 2 mL of a 0.5 M Tris-HCl (pH 7.6)-1.5 M NaCl solution, and left for 10 min. The cellulose filter was removed and left at ambient temperature for 30 min to dry. The denatured DNA of the colonies was fixed to the surface of the cellulose filter by UV irradiation of 254 nm at a 10 cm distance for 5 min [100 ergs.$(mm^2)^{-1}.s^{-1}$]. The cellulose filters were placed, back to back, in 18 oz Whirl-Pak bags, containing 6 X SSC-5 X Denhardt's solution-0.5% SDS (pre-hybridization solution; 7 mL) and incubated in a water bath at 68°C for 2 h with gentle shaking. The pre-hybridization solution was replaced by 6 X SSC-5 X Denhardt's (hybridization solution; 7 mL), containing the freshly-denatured ^{32}P-labelled probe DNA (1 µg; 1 X 10^6 cpm). The incubation in a 68°C water bath with gentle shaking was continued overnight. The radioactive probe was recovered by pouring into a sterile 15 mL conical centrifuge tube. The tube was screw-capped and stored at -20°C. The cellulose filters were washed 3 times for 15 min each in 2 X SSC-0.5% SDS (30 mL) at 68°C with vigourous shaking and air dried. They were placed in the correct orientation in a cassette fitted with HI+ intensifying screens and containing X-ray film. The cassette was locked and placed at -70°C for about 5 h. The film was developed for 2 min, washed in running tap water for 2 min, fixed for 4 min, then examined for darkened areas which were compared to the inoculation pattern on the original agar plate.

2.10.3 Colony Hybridization on HGMFs

Preparation of HGMF for colony hybridization. The HGMF was inoculated by replication (see Sect. 2.11), or by filtration, then incubated overnight at 30° or 37°C as required. The HGMF was placed for 30 min on filter paper which had been soaked with 5 mM sodium phosphate (pH 6.0)-100 mM sodium bicarbonate-0.0066% PEI (pre-treatment solution; 2.5 mL), air-dried for 10 min, and placed on filter paper which had been soaked with 150 mM NaOH in 70% ethanol (lysis solution; 2.5 mL). After exposure to microwave irradiation of ≈ 700 W for 30 s, the HGMF was placed in a small plastic box containing 0.01% proteinase K-2 X SSC-0.1% SDS (20 mL), covered with a lid, and placed in a water bath at 37°C for 1 h, with gentle shaking. It was washed for 5 min in each of 2 X SSC-0.1% SDS (30 mL), and 2 X SSC (30 mL), then air-dried for 30 min before the denatured DNA was fixed to the membrane by exposure to UV irradiation as before. Denatured bacterial growth on HGMFs prepared this way can be stored at room temperature in air for 6 mo, or under vacuum for up to 1 y, for future testing with probes.

Use of radiolabelled probe. The HGMF, treated as described above, and carrying denatured, fixed DNA, was placed in an 18 oz Whirl-Pak bag containing pre-hybridization solution (7 mL), and incubated in a 68°C water bath for 2 h with gentle shaking. The pre-hybridization solution was replaced with hybridization solution (7 mL) containing denatured ^{32}P-labelled probe DNA (1 μg; 1 X 10^6 cpm), prepared as previously described, and incubation was continued overnight under the same condition. Washing and autoradiography of the HGMF were carried out as described for cellulose filters. Dark spots on the developed film were compared to the inoculation pattern of the HGMF.

Use of chromogen-labelled probe. The hybridization procedure was a modification of the method of Downs et al. (1987). The HGMF, treated as described above, and carrying denatured, fixed DNA, was placed in an 18 oz Whirl-Pak bag containing 50% formamide-2 X Denhardt's-12%PEG 8000-0.1% SDS (chromogen hybridization solution; 7 mL) and incubated in a water bath at 38°C for 2 h, with gentle shaking. Thirty-six μL of HRP-labelled DNA probe, prepared as

described above (Sect.2.9.2), were added directly to the hybridization solution containing the HGMF, the bag was re-sealed, and the incubation continued overnight. The HGMF was washed for 2 X 30 min at 38°C, with vigourous shaking, in 50% formamide-1 X SSC-0.5% SDS, and for 2 X 30 min at room temperature, with gentle shaking, in 1 X SSC. Colour development solution was prepared by mixing a solution of 4-chloro-1-naphthol (60 mg) in cold methanol (20 mL), and adding it to 20 mM Tris-HCl (pH 7.5)-500 mM NaCl (100 mL) containing 60 µL of 30% hydrogen peroxide. The presence of HRP was detected by placing the HGMF for up to 1 h in colour development solution (15 mL), yielding a purple precipitate at the site of the reaction.

Alternatively, Dig-labelled probe, prepared as described above (Sect 2 9.2) was used. The HGMF, treated for cell lysis and DNA denaturation as described above, was placed in an 18 oz Whirl-Pak bag. Hybridization, washing and colour development were performed according to the manufacturer's procedure already described (Boehringer Mannheim, 1988; Sect 2.10 1).

2.11 Deployment and Replication of Organisms on HGMFs

The plasmid DNA of each of the 7 hemolytic clones was screened against the DNA of an array of organisms. To accomplish this, a master HGMF holding either 40 or 100 organisms was prepared and replicated as described in Peterkin et al. (1989c). For testing the ^{32}P-labelled probes, 40 organisms, consisting of 10 L. monocytogenes strains (Nos. 1-10, Table 2-1), 10 other Listeria spp. (Table 2-2) and 20 organisms of other genera (Table 2-3) were used (PROBESCREEN40). For later testing of the chromogen-labelled probe, 60 additional L. monocytogenes strains (Nos. 41-100, Table 2-1) were added to form a 10 X 10 array of organisms (see Fig. 3-11; PROBESCREEN100). In both cases, the organisms were manually deployed on a master HGMF with the aid of "PUT", a computer program for the HGMF Interpreter (Sharpe et al., 1989). An HGMF was laid in a square depression in a plate beneath the Interpreter's TV camera (Fig. 2-2). To prevent contamination of the HGMF surface, the depression was covered

Table 2-1. L. monocytogenes strains used for screening the DNA probes

No.	Strain[a]	Serovar	No.	Strain	Serovar
1.	V7	1c	66.	83-427	4a
2.	1472-1	1/2	67.	83-641	1/2
3.	19112	1/2c	68.	83-648	4b
4.	19113	3	69.	83-649	1/2
5.	LA-5	4	70.	83-937	4b
6.	81-861	4b	71.	83-5067	4b
7.	19116	4c	72.	83-9138	1/2
8.	85-362	4d	73.	83-10657	1/2
9.	19118	4e	74.	83-10984	4b
10.	82-26	4ab	75.	84-13	1/2
41.	F4968	4b	76.	84-103	1/2
42.	F4989	ND[b]	77.	84-108	1/2
43.	F6861	4b	78.	84-109	1/2
44.	F7032	ND	79.	84-110	1/2
45.	19117	4d	80.	84-153	1/2
46.	DA-2	4	81.	84-251	ND
47.	DA-3	4	82.	84-331	1/2
48.	V37CE	ND	83.	84-362	1/2
49.	Murray B	ND	84.	84-434	1/2
50.	Scott A	4b	85.	84-542	1/2
51.	82-129	4b	86.	84-607	4b
52.	82-130	4b	87.	84-2393	ND
53.	82-131	4a	88.	85-11	4b
54.	82-132	4b	89.	85-190	4b
55.	82-204	1/2	90.	85-206	1/2
56.	82-335	1/2	91.	85-231	1/2
57.	82-336	1/2	92.	85-232	1/2
58.	82-338	1/2	93.	85-263	1/2
59.	82-339	1/2	94.	85-307	1/2
60.	82-340	1/2	95.	85-416	4d
61.	82-341	1/2	96.	85-429	1/2
62.	82-342	4ab	97.	85-442	1/2
63.	82-464	1b	98.	85-492	4b
64.	82-465	1a	99.	85-496	1/2
65.	83-276	4b	100.	85-513	1/2

[a] All strains were obtained from the collection maintained at Health Protection Branch, Ottawa, Ontario

[b] ND - not determined.

Table 2-2. Listeria species other than L. monocytogenes used for screening the DNA probes.

No.	Organism	Strain	Source[a]
11.	L. innocua	LA-1	HPB
12.	L. innocua	29	HPB
13.	L. welshimeri	160H1-3	HPB
14.	L. seeligeri	47295μA	HPB
15.	L. seeligeri	3293	HPB
16.	L. ivanovii	LA-2	HPB
17.	L. ivanovii	LA-3	HPB
18.	L. grayi	19120	ATCC
19.	L. murrayi	25401	ATCC
20.	L. denitrificans[b]	14870	ATCC

[a] HPB - Health Protection Branch, Ottawa, Ontario; ATCC - American Type Culture Collection, Rockville, MD.

[b] Reclassified as Jonesia denitrificans (Rocourt et al., 1987).

Table 2-3. Organisms other than Listeria spp. used for screening the DNA probes.

No.	Organism	Strain	Source[a]
21.	Erysipelothrix rhusiopathiae	75-833	HPB
22.	Lactobacillus casei (rham)	84-121	HPB
23.	Brochothrix thermosphacta	11 509	ATCC
24.	Corynebacterium aquaticum	73-958	HPB
25.	Arcanobacterium haemolyticum	76-763	HPB
26.	Oerskovia spp.	80-44	HPB
27.	Kurthia zopfii	33403	ATCC
28.	Escherichia coli	25922	ATCC
29.	Citrobacter freundii		HPB
30.	Klebsiella pneumoniae		HPB
31.	Salmonella johannesburg		HPB
32.	Enterobacter aerogenes		HPB
33.	Pseudomonas aeruginosa		HPB
34.	Aeromonas hydrophila		HPB
35.	Staphylococcus aureus (SED)	23235	ATCC
36.	Bacillus cereus	H30	HPB
37.	Yersinia entercolitica	Y28 0:9	HPB
38.	E. coli DH5∝		HPB
39.	Vibrio vulnificus	2	HPB
40.	Gemella haemolysans	10379	ATCC

[a] HPB - Health Protection Branch, Ottawa, Ontario; ATCC - American Type Culture Collection, Rockville, MD.

Fig. 2-2. HGMF Interpreter showing "Putting". Below the TV camera can be seen the HGMF to be inoculated, covered by a transparent plastic square with a 2.5 mm hole in its centre The hole is positioned over the target grid cell which is flagged on the camera monitor. The exposed grid cell is inoculated with a toothpick dipped into a broth of the required culture.

with a plastic sheet containing a small hole through which the inoculation was done. Computer program PUT recognized the HGMF image, calculated the position of every grid-cell, and, in succession, illuminated on the TV monitor every 4th grid-cell. These grid-cells were inoculated manually in the desired order, using a sterile toothpick, with overnight cultures in BHI broth of the organisms. The master HGMF, thus inoculated, was incubated overnight at 30°C, in order to allow growth both of B. thermosphacta and the other organisms.

The HGMF Replicator was then used to make as many copies of PROBESCREEN40 or PROBESCREEN100 as were required. The uninoculated slave HGMFs were placed on BHI agar plates. Inoculators with 100 (10 X 10) points were used, and were reloaded after every 5 copies. The master HGMF was moved by hand until the cross-hairs in the instrument's eyepiece aligned to the HGMF border marker lines. The inoculator was lowered momentarily to the surface of the HGMF by means of compressed air. The master HGMF was withdrawn and replaced in succession by the slave HGMFs, and the cycle was repeated. The replicates were incubated overnight at 30°C for immediate use in screening the plasmid or insert DNA for specificity and sensitivity. For long-term storage, PROBESCREEN100 was replicated to HGMFs on plates of BHI agar containing 20% glycerol. The plates were incubated at 30°C for 6 h, wrapped in plastic sleeves, and stored at -78°C. Plates were removed at monthly intervals and, after standing on the bench for 2 h, the HGMFs were transferred to BHI plates. After overnight incubation at 30° C, the HGMFs were examined for growth of the organisms of PROBESCREEN100.

2.12 Detection of L. monocytogenes in Foods by the HGMF-based DNA Probe Method

Five samples of each of three foods (soft cheese, raw milk, ground chicken) were separately inoculated with either L. monocytogenes (strain 81-861), or one of three other Listeria spp. (L. innocua LA-1; L. welshimeri 160H1-3; L. seeligeri 472595μA. The inoculation levels used were either 1 or 100 Listeria cells.g^{-1} food. The presence of L. monocytogenes was detected in these samples, and in the uninoculated foods (a total of 135 determinations), by the conventional

method appropriate to the food, or by the newly-developed HGMF-based DNA probe method. The results obtained by the two methods were compared.

HPB procedure #MFLP-59 (HPB, 1988a) was used for soft cheese (Esrom, Denmark) and raw milk. A food sample (25 g) inoculated with either 1 or 100 cells of the required organism grown overnight in BHI broth, or a 25 g sample of uninoculated food, was added to enrichment broth (EB; 225 mL), blended in a Stomacher for 60 s, and incubated for 24 h at 30°C in the Stomacher bag. LPM and Oxford agar plates, and a tube of Fraser broth (5 mL), were each inoculated with a loopful of the incubated broth. At the same time, HGMF were inoculated by vacuum filtration with 1 mL of the incubated broth and laid on the surface of LPM or Oxford agar plates. The plates were incubated for 24-48 h, the LPM plates at 30°C, and the Oxford plates at 35°C. The tube of Fraser broth was incubated at 35°C. If after 24 h, the Fraser broth appeared darkened from growth of Listeria spp., LPM and Oxford agars were each inoculated with a loopful of the broth, and incubated as before.

HPB procedure #MFLP-60 (HPB, 1988b) was used for ground chicken. A food sample (25 g) inoculated with either 1 or 100 cells of the required organism grown overnight in BHI broth, or a 25 g sample of uninoculated food, was added to Listeria enrichment broth (LEB; 225 mL), blended in a Stomacher for 60 s, and incubated overnight at 30°C in the Stomacher bag. Incubated broth (0.1 mL) was transferred to a tube of Fraser broth (5 mL), which was incubated overnight at 35°C. If the Fraser broth darkened (positive reaction), LPM and Oxford agar plates were each inoculated with a loopful of the broth, and at the same time, HGMF were inoculated by vacuum filtration with 1 mL of the broth and laid on the surface of LPM or Oxford agar plates. The plates were incubated for 24 h, the LPM plates at 30°C, and the Oxford plates at 35°C.

Typical Listeria colonies on LPM agar are small, and bluish by oblique transillumination. Typical colonies on Oxford agar are black, and surrounded by black haloes. Five typical colonies from each plate were inoculated onto trypticase soy agar (Difco) with 0.6% yeast extract (TSAYE), and incubated for 24 h at 30°C. An isolated colony was inoculated into 7% horse blood agar,

motility medium (Difco), and xylose and rhamnose agars, and incubated for 24 h at 35°C. A Gram stain and a test for the presence of catalase were also performed. L. monocytogenes is a small Gram-positive motile rod which is ß-hemolytic, shows catalase activity, and ferments rhamnose but not xylose.

The HGMFs containing bacterial growth were prepared for hybridization as described above (Sect. 2.10.3). Dig-labelled pIP5 DNA was prepared as previously described (Sect. 2.9.2), and DNA/DNA hybridization and washing were carried out as described in Sect 2.10.3. The presence of homology to the probe was detected by an AP-labelled anti-Dig antibody which, when reacted with the substrate NBT/BCIP, formed a blue precipitate at the site of the hybridization (Sect. 2.10.1).

3. RESULTS

3.1 L. monocytogenes Plasmids

One hundred and twenty-two ß-hemolytic strains of L. monocytogenes were analyzed for the presence of plasmids. Twenty-five of the strains (20%) contained plasmids, with molecular sizes ranging from 2.0 to 100 kb (Table 3-1). Of 11 strains of other Listeria spp. examined [3 L. innocua, 2 L. ivanovii, 2 L. welshimeri, 2 L. seeligeri, 1 each of L. murrayi, and L. grayi], 7 were found to contain plasmids ranging from 58 to 71 kb (Table 3-1). L. denitrificans (J. denitrificans) was also analyzed; no plasmid was isolated. Multiple plasmids were not observed.

3.2 L. monocytogenes Chromosomal DNA

In initial experiments to optimize the recovery of DNA from L. monocytogenes cells, the procedure of Hadley and Deonier (1979) was modified by increasing the lysozyme incubation time to 60 min, and by substituting 50 mM Tris-HCl (pH 7.5)-62.5 mM EDTA-2.5 M LiCl-0.4% Triton X-100 buffer (TELT; Wilimzig, 1985) for Triton X-100 and DNase-free RNase incubation. The modified procedure, in which the RNA was precipitated by the LiCl, resulted in an improved lysis as shown by higher viscosity and greater clarity of the solution of lysed cells. Following the CsCl density gradient centrifugation, three succesive preparations of chromosomal DNA from L. monocytogenes strain 81-861 cells, yielded from 17 to 20 µg of DNA.mL^{-1} of broth culture. The DNAs from these preparations showed an OD_{260}/OD_{280} ratio that ranged from 1.80 to 1.84, agarose gel electrophoresis showed neither smearing from low molecular size DNA, nor the presence of RNA (Fig. 3-1).

Chromosomal DNA from two of the preparations was tested for the presence of nucleases active under conditions of either restriction endonuclease digestion, or ligation with T4 DNA ligase. Chromosomal DNA (4 µg) was incubated in the presence of 20 mM Tris-HCl (pH 7.5)-10 mM $MgCl_2$ for either a) 2 h at 35°C (restriction digest conditions), or b) 14 h at 16°C (ligation

Table 3-1. Plasmids in L. monocytogenes and other Listeria species

Species	Strain	Serovar	Collection*	Source	Plasmid size (kb)
L. monocytogenes	82-129	4b	HPB	stool	2.0
"	82-130	4b	HPB	stool	2.0
"	82-132	4b	HPB	stool	2.0
"	84-109	1/2	HPB	vagina	57
"	84-110	1/2	HPB	cervix	57
"	85-206	1/2	HPB	clinical	57
"	85-514	1/2	HPB	blood	57
"	CY2-286		LSPQ	clinical	57
"	CY2-1227	1/2	LSPQ	clinical	57
"	84-108	1/2	HPB	posterior fornix	63
"	84-153	1/2	HPB	blood	63
"	85-263	1/2	HPB	CSF	63
"	85-492	4b	HPB	blood	63
"	CY0-83	1/2	LSPQ	clinical	63
"	CY2-920	1/2	LSPQ	clinical	63
"	CY2-287	4b	LSPQ	clinical	70
"	CY2-1208		LSPQ	clinical	70
"	CY2-636	1/2	LSPQ	clinical	91
"	84-362	1/2	HPB	blood	100
"	84-542	1/2	HPB	blood	100
"	1297	1/2	HPB	cheese	61
"	1472-1	1/2	HPB	cheese	65
"	397	4b	HPB	alfalfa tablets	65
"	12308N86		ADRI	bovine brain	60
"	84-2393		ADRI	ovine	74
L. innocua	124			meat	65
L. innocua	LA-1	4		meat	58
L. innocua	29	4		meat	80
L. welshimeri	160H1-3	6b			58
L. seeligeri	472595μA				71
L. ivanovii	LA-2	5		raw cream	71
L. ivanovii	LA-3	5		raw cream	71

*HPB - Health Protection Branch, Ottawa, Ontario; ADRI - Animal Diseases Research Institute, Nepean, Ontario; LSPQ - Laboratoire de Santé de la Province de Québec, Sennevill9, Québec

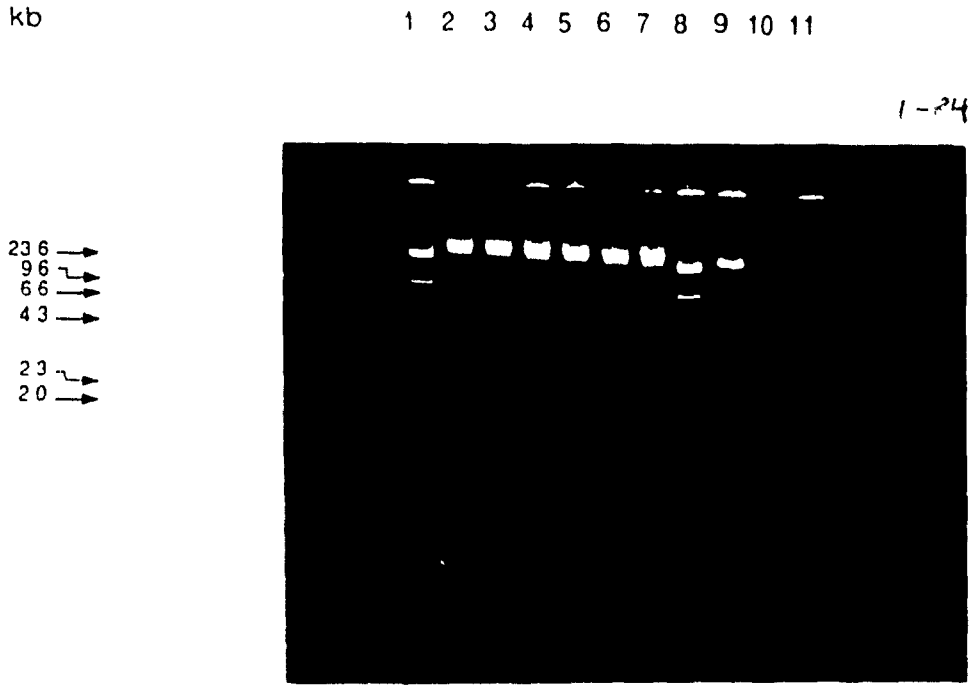

Fig. 3-1. Listeria monocytogenes 81-861 chromosomal DNA. Lanes 2-7, 9-11: L. monocytogenes DNA samples from replicate tubes of one preparation; lanes 1 and 8: molecular size markers of λ phage DNA Hind III fragments.

conditions). There was no increase in low molecular size DNA during these incubations, when compared to non-incubated controls (results not shown).

3.3 Preparation and Storage of Genomic Libraries

In preliminary experiments, competent E. coli JM83 and JA221 cells were prepared by three recognized methods, and their transformation rates with CCC pUC18 (50 ng) compared (Table 3-2). Method 1 uses both $MgCl_2$ and $CaCl_2$ chilled solutions in preparing competent cells, Method 2 uses only $CaCl_2$ solution, and Method 3 uses both $MnCl_2$ and $CaCl_2$ solutions. Method 1 gave a two- to sixfold greater transformation efficiency than the other two methods, and was used in the preparation of cells for early transformations.

For later transformations, Hanahan's method of preparing competent cells was used. This method, employing a 2-N-morpholinoethane sulfonic acid buffer containing $MnCl_2$, $CaCl_2$, RbCl, and hexamine cobalt (III) trichloride ions, was tested with E. coli DH5 cells (Hanahan, 1983). The claimed transformation rate of 5×10^6 transformants.μg^{-1} of plasmid DNA was confirmed.

Preparation. The first genomic library of L. monocytogenes 81-861 chromosomal DNA (GL1) was prepared in BamH I-cut pBR322 [1 μg of vector DNA (V)] using Mbo I - generated genomic fragments from 8 to 18 kb in size [2 μg of insert DNA (I)], at a molar ratio of I/V=1 (Peterkin and Idziak, 1986). This was used to transform competent cells of host strain E. coli C600 with a transformation frequency of 10^3 transformants.μg DNA^{-1}. The transformants were inoculated by filtration, at a level calculated to deposit about 70 cells per filter, onto 40 HGMFs. After overnight incubation on TSA Ap_{50} a total of 2800 ampicillin-resistant clones were obtained with a known probability of purity of 98% (Table 3-3). Replicates of these HGMFs, incubated overnight on TSA Tet_{15} plates, showed about 10 tetracycline-resistant transformants per filter (Fig. 3-2). Thus each filter carried 60 tetracycline-sensitive transformants for a total of 2400 (86%) clones with insertional inactivation.

Two test genomic libraries, GL2 and GL3, were then prepared in E. coli DH5 or E. coli DH5α host cell strains, using plasmid vectors pBR322 and pUC18 respectively. GL2 consisted

Table 3-2. Comparison of methods for preparing competent cells

Host cell strain	Method		
	1[a]	2[b]	3[c]
	(Transformants . μg DNA^{-1} x 10^{5})		
JM83	2.4	1.4	0.5
JA221	3.7	0.6	0.8

[a] Dillon et al., 1985
[b] Maniatis et al., 1982
[c] MacLeod et al., 1985

Table 3-3. Probability of purity[a] as a function of number of positive grid cells on ISO-GRID HGMF

X	P	X	P
1	.9997	160	.9482
2	.9994	170	.9449
5	.9985	180	.9415
10	.9969	190	.9381
20	.9937	200	.9347
30	.9906	210	.9313
40	.9874	220	.9279
50	.9842	230	.9244
60	.9810	240	.9209
70	.9778	250	.9175
80	.9746	260	.9140
90	.9713	270	.9104
100	.9681	280	.9069
110	.9648	290	.9033
120	.9615	300	.8998
130	.9582	350	.8816
140	.9549	400	.8630
150	.9516		

$$^{a}P = -\frac{N-X}{X} \ln \frac{N-X}{N}$$

Where P = probability of purity
N = total no. of grid cells (1600 for ISO-GRID HGMF)
X = no. of positive grid cells

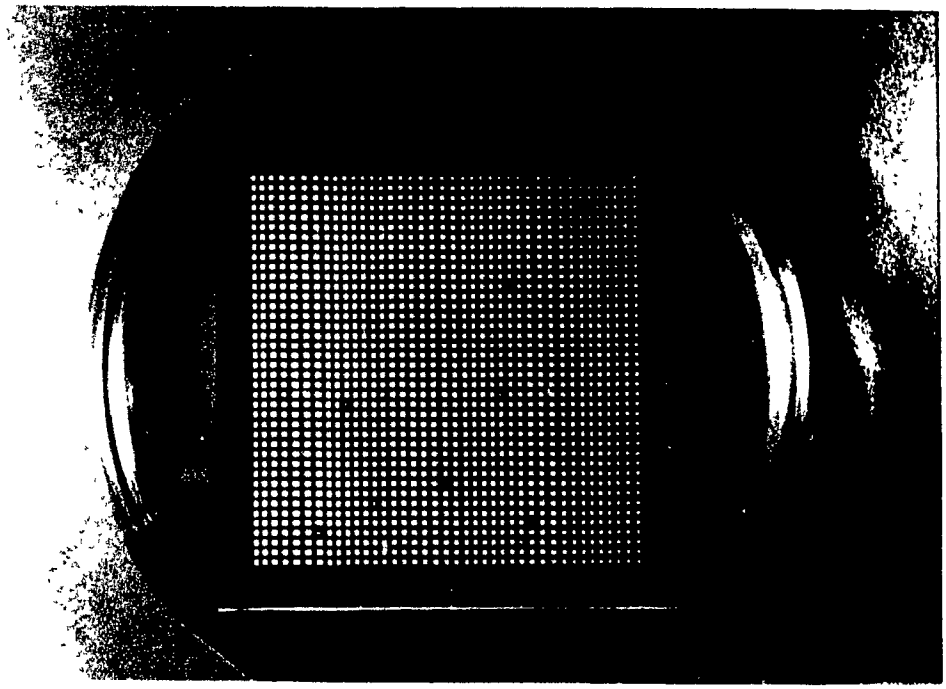

Fig. 3-2. Tet^R transformants on HGMF. Transformants replicated from an HGMF containing about 60 ampicillin-resistant clones, and grown on tryptose soy agar plates containing tetracycline at 15 $\mu g.mL^{-1}$.

of Mbo I-cut L. monocytogenes 81-861 chromosomal DNA fragments (not size-separated), ligated in BamH I-cut pBR322 and used to transform DNA E. coli DH5 competent cells. No hemolysis was observed when 320 Ap^R Tet^S clones of GL2 were tested on 7% horse blood agar. In preparing GL3, Mbo I-cut L. monocytogenes chromosomal DNA fragments (5-10 kb) were ligated in BamH I-cut pUC18 DNA, and the ligation mixture was used to transform E. coli DH5α host cells, at a transformation frequency of 3 X $10^5.\mu g^{-1}$ DNA. Agarose gel electrophoresis of recombinant plasmids showed an average insert size of about 2 kb, with an insertional inactivation rate of 12%.

The genomic library (GL4) which formed the basis of the research reported here was prepared using L. monocytogenes 81-861 Mbo I - generated DNA fragments from 8 to 12 kb in size, ligated into BamH I-cut pUC18 DNA. The resulting recombinant plasmids were used to transform competent cells of host strain E. coli DH5α, at a transformation frequency of 6 X 10^6 transformants.μg^{-1} DNA (transformation frequency of CCC pUC 19 monomer used as a control = 1 X 10^8 transformants.μg^{-1} DNA). A total of 1.9 X 10^4 transformants showing insertional inactivation by the production of colourless colonies when plated on X-gal-containing medium, were obtained. Agarose gel electrophoresis of recombinant plasmid DNA from 12 GL4 clones showed sizes ranging from 4.5 to 15.5 kb, indicating inserts of about 1.8 to 12.8 kb (Fig. 3-3). Assuming an average insert size of 8 kb, 2300 transformants gave a 99% probability of cloning all the chromosomal genes, if the L. monocytogenes genome is the same size as that of E. coli (Clarke and Carbon, 1976).

Storage of GL4. After three years of storage at -70°C, the cells kept in 20% glycerol (Method 2) remained viable. The viability of cells deployed on HGMFs and stored on agar containing 20% glycerol (Section 2.4, Method 3; Fig. 3-4) has been demonstrated after two years of storage.

3.4 Identification and Characterization of ß-Hemolytic Clones

ß-Hemolytic activity of recombinant clones. During screening of 2500 clones of the L. monocytogenes gene bank GL4, expression of the ß-hemolysin determinant was demonstrated

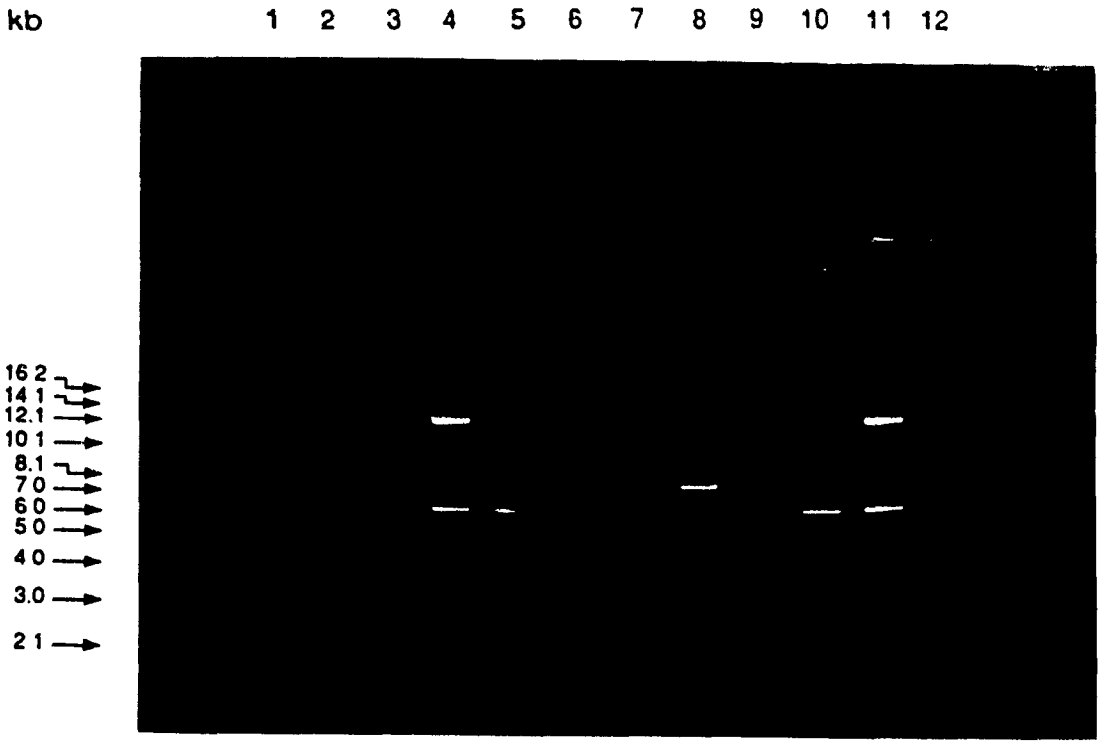

Fig. 3-3. Molecular sizes of a selection of recombinant plasmids of GL4. Lanes 2-11: recombinant plasmids of genomic library #4 showing molecular sizes ranging from 4.5 to 15.5 kb; lanes 1 and 12: CCC plasmid DNA molecular size markers ranging from 2.1 to 16 kb.

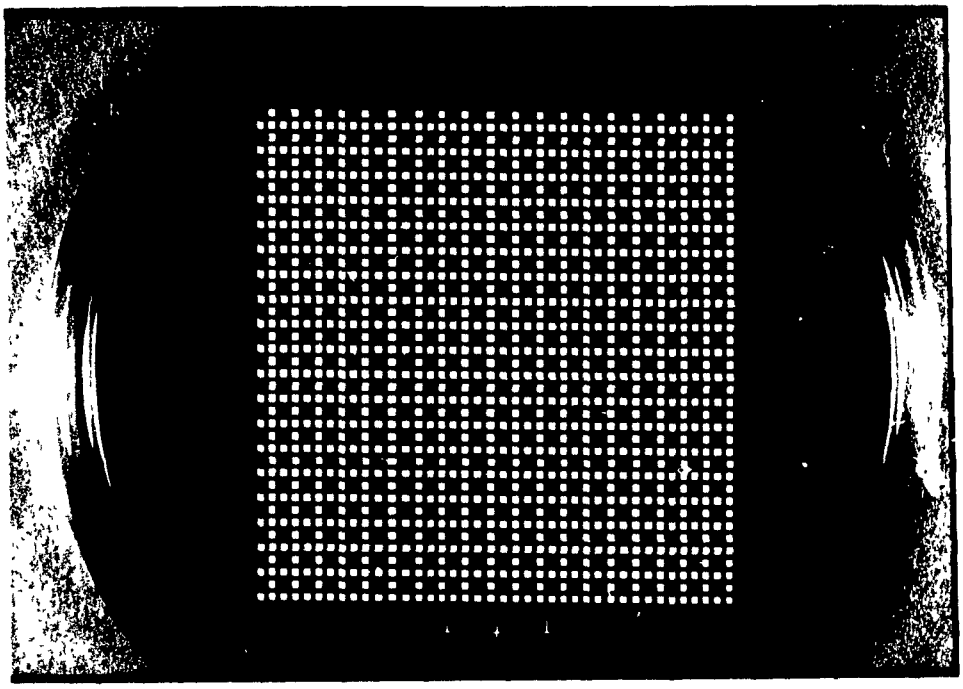

Fig. 3-4. Storage of 400 clones in an ordered array on HGMF. Several copies of clones of genomic library #4, inoculated and replicated as described, were incubated on BHI agar containing 20% glycerol and stored at -70°C. The 2500 screened clones were stored on 7 HGMFs. Culture locations are defined as (row, column) starting at the upper left hand grid cell (1,1).

in 7 clones (0.3%) by a zone of clearing surrounding each colony growing on 7% horse blood agar. The purity of the 2500 selected clones was demonstrated by colourless growth when inoculated to LB_{AX} agar plates and incubated overnight at 35°C. The appearance of these clones when stabbed into a 7% horse blood agar plate, together with L. monocytogenes and L. innocua positive and negative controls, is shown in Fig. 3-5. Six of the 7 clones show a zone of hemolysis approaching that of L. monocytogenes 81-861 in size, but the hemolytic activity shown by clone 39-9 is weak. The recombinant plasmids of the 7 ß-hemolytic clones were named pIP1 to pIP7. However, E. coli DH5α competent cells which were retransformed with plasmid DNA isolated from the hemolytic clones, demonstrated expression of ß-hemolytic activity only with clones containing plasmids pIP1, pIP2, pIP3, pIP5, or pIP7 (Fig. 3-6). Subsequent work on the characterization of these clones and of their plasmids, was done on these 5 recombinant strains only (Table 3-4).

When hemolytic activity was measured in the microtitre plate assay, broth cultures of each of the 5 clones demonstrated titres ranging from 1 to a minimum of 8 units after 48 h incubation; the addition of 6 mM cysteine did not change the titre (Table 3-5). The L. monocytogenes 81-861 control culture showed a minimum activity of 8 units.

Test of cell fractions for hemolytic activity. The hemolytic activities of the culture supernatants of the 5 ß-hemolytic clones, as well as of control strains, were assayed in microtitre plates. Of the test and control strains examined, only the L. monocytogenes supernatant showed hemolytic activity (Table 3-5). Extracts of broken cells prepared in the following ways (with and without the addition of 6 mM cysteine) were also tested: sonicated cell extracts, their supernatants and their resuspended pellets, and cell lysates. With all types of cell fractions, only the L. monocytogenes 81-861 control preparation showed hemolytic activity (results not shown).

CAMP test. Two of the hemolytic clones, E. coli DH5α (pIP2 and pIP7), and L. monocytogenes 81-861, demonstrated an enhanced ß-hemolysis when grown in the presence of S. aureus (Table 3-5). The hemolytic zones of the remaining clones were not enhanced.

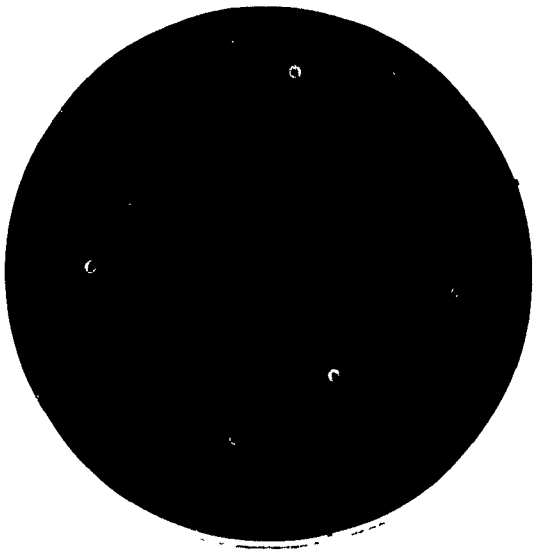

Fig. 3-5. Growth of hemolytic recombinant clones on 7% horse blood agar showing zones of ß-hemolysis, after overnight incubation at 35°C. 81-861: L. monocytogenes 81-861 (positive control); LA-1: L. innocua LA-1 (negative control); 4-8, 10-37, 26-3, 27-6, 34-3, 39-9, 46-2S: isolation numbers of ß-hemolytic clones containing plasmids pIP1 to pIP7.

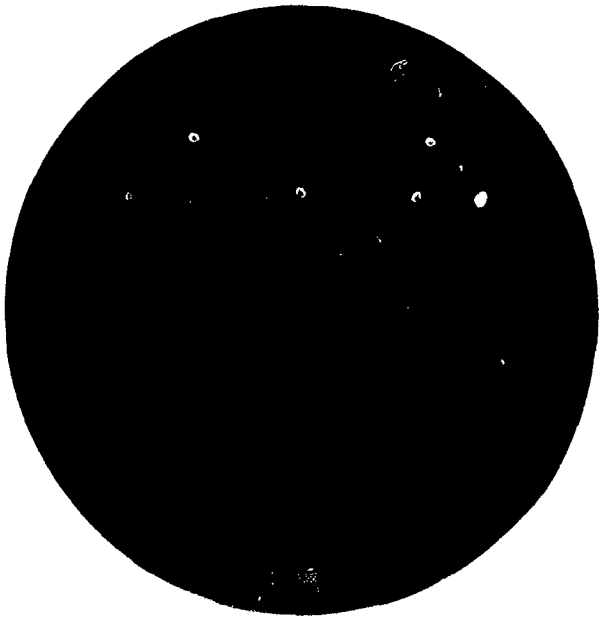

Fig. 3-6. Expression of ß-hemolytic activity by clones after retransformation. E. coli DH5α cells were tranformed with plasmid DNA pIP1 to pIP7, isolated from the ß-hemolytic clones. Growth of the retransformed clones on 7% horse blood agar, after overnight incubation at 35°C, is shown. L.m.: L. monocytogenes 81-861 (positive control); L.i. and Ec (pUC18): L innocua LA-1 and E. coli DH5α (pUC18) (negative controls); pIP1 to pIP7: E. coli DH5α clones retranformed with plasmids pIP1 to pIP7.

Table 3-4. Molecular sizes of hemolytic recombinant plasmids of L. monocytogenes gene bank.

Isolate number	Plasmid	Molecular weight	
		Plasmid (bp)	Insert (bp)
4-8	pIP1	6040	3350
10-37	pIP2	7030	4340
26-3	pIP3	3690	1000
34-3	pIP5	4300	1610
46-29	pIP7	4130	1440

Table 3-5. Hemolytic activity and virulence of β-hemolytic clones of L. monocytogenes gene bank.

Species/Strain	Hemolysin			Mouse lethality		CAMP	Cytolysin
	Cells		Supernatant	Experiment #1	Experiment #2		Supernatant
E. coli DH5∝ (plp1)[a]	+[e]	2[f]	-[g]	5/5[h]	2/5	-	$+_w$[i]
E. coli DH5∝ (plP2)[a]	+	2	-	3/5	3/5	+	-
E. coli DH5∝ (plP3)[a]	+	1	-	5/5	2/5	-	+
E. coli DH5∝ (plP5)[a]	+	4	-	5/5	5/5	-	$+_w$
E. coli DH5∝ (plP7)[a]	+	≥8	-	3/5	4/5	+	$+_w$
E. coli DH5∝ (plP8)[b]	-	-	-	0/5	0/5	-	-
E. coli DH5∝ (plP9)[b]	-	-	-	0/5	0/5	-	-
L. monocytogenes 81-861	+	≥8	≥8	5/5	5/5	+	4+
L. innocua LA-1	-	-	-	0/5	0/5	-	-
E. coli DH5∝[c]	-	-	-	0/5	0/5	-	-
E. coli DH5∝ (pUC18)[d]	-	-	-	0/5	0/5	-	-

[a]Hemolytic recombinant. [b]Non-hemolytic recombinant. [c]Host organism of gene bank. [d]Host organism with plasmid vector. [e]As observed after stabbing cells into 7% horse blood agar, scored as follows: +, hemolytic; $+_w$, weakly hemolytic; -, non-hemolytic. [f][g]Reciprocal of highest dilution of [f]broth culture and [g]culture supernatant to cause complete hemolysis of sheep red blood cells in the microtiter plate assay. [h]Number of mice killed/number of mice injected with $\approx 10^9$ cells, observed over a 7 day period. [i]Lysis of CHO cells observed with filtered culture supernatant in a microtiter well, scored on a scale of $+_w$ to 4+.

L. innocua LA-1, E. coli DH5α, E. coli DH5α (pUC18) and two non-hemolytic clones showed no hemolysis when grown on the sheep blood agar plates of the CAMP test.

Cytolytic activity of filtered supernatants. Weak cytolytic activity against CHO cells was shown by filtered supernatants of 4 of the 5 hemolytic clones, E. coli DH5α (pIP1, pIP3, pIP5, or pIP7) (Table 3-5). Marked cytolysis of the CHO cells was shown only by the L. monocytogenes 81-861 supernatant. Filtered supernatants of all other test and control strains examined showed no cytolytic activity.

3.5 Pathogenicity of β-Hemolytic Clones

Mouse lethality testing. In duplicate experiments, intraperitoneal injection of approx. 10^9 cells of each of the five hemolytic recombinants resulted in the death of from 2/5 to 5/5 mice (Table 3-5). All of the deaths occurred during the first 3 days of the 7-day observation period. All five mice were killed by L. monocytogenes 81-861 cells, whereas cells of L. innocua LA-1, E. coli DH5α, E. coli DH5α (pUC18) and two non-hemolytic clones were non-lethal to mice. Tests for β-hemolysis made by stabbing into horse blood agar plates concurrently with the inoculation of the initial cultures for these experiments, showed that the determinant for β-hemolysin was being expressed in all 5 hemolytic clones.

3.6 Characterization of Insert DNA of β-Hemolytic Clones

Insert molecular size. Plasmids from the 5 clones showing β-hemolytic activity after retransformation, namely pIP1, pIP2, pIP3, pIP5, and pIP7, have molecular sizes ranging from 3690 to 7030 bp. As the pUC18 vector is 2690 bp in size, the molecular sizes of the inserts vary from 1000 to 4340 bp (Table 3-4).

Restriction maps of Insert DNAs. Restriction digests of the plasmid DNA from the hemolytic clones showed that pIP1 and pIP2 contained a 650-bp Hind III fragment not seen in the other inserts (Fig. 3-7). Restriction maps of the insert DNA from recombinant plasmids pIP1, pIP2,

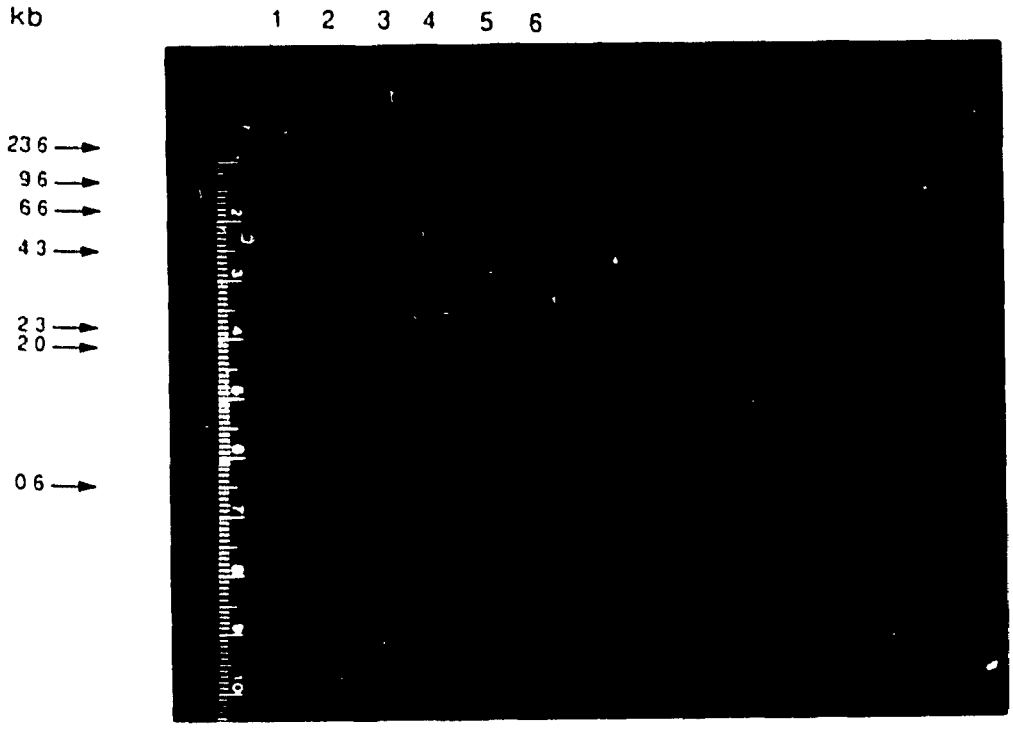

Fig. 3-7. Hind III digests of plasmid DNA of hemolytic recombinant clones. Lanes 2-6: Hind III digests of pIP1, pIP2, pIP3, pIP5, and pIP7 DNA; lane 1: molecular size markers of λ phage DNA Hind III fragments.

pIP3, pIP5, and pIP7, were constructed (Fig. 3-8; Appendix A). Deduced fragment sizes of double digests with Hind III/Kpn I and with Hind III/Pvu II were confirmed experimentally (Appendix B)

Southern hybridizations. In order to determine whether homology exists amongst the insert DNAs, digests of the plasmid DNA of the 5 hemolytic recombinant clones were probed with labelled DNA of pIP1, pIP2, pIP5, or pIP7. The plasmid DNA was digested with Hind III and subsequently with Kpn I, separated by agarose gel electrophoresis (Fig 3 9), and transferred to a Nytran membrane. After hybridization with digoxigenin-labelled pIP1, pIP2, pIP5, or pIP7 DNA, and visualization of the chromogen, the Southern blots had the appearance shown in Figs 3-10 to 3-13. All of the probes hybridized to the 2 7 kb bands of pUC18 vector DNA (see Fig 3-9) However, none of them hybridized to bands of insert DNA other than their own, indicating a lack of homology between the inserts of these 5 recombinant plasmids.

When a similar Southern blot of a Hind III/Kpn I double digest of the plasmid DNAs of the hemolytic recombinant clones was probed with digoxigen-labelled pAD319 DNA, and the chromogen visualized, the result shown in Fig 3-14 was obtained This plasmid contains about 3.1 kb of L. monocytogenes Scott A strain DNA, including the hlyA gene, in pUC8 (Datta et al , 1990). It hybridized to the 2.7 kb bands of pUC18 vector DNA of all 5 hemolytic recombinant clones. As well, homology was demonstrated with the insert DNA of pIP2, as pAD319 hybridized to the 3000, 650 and 300 bp bands, though not with the 600 bp band. No homology was seen with the inserts of pIP1, pIP3, pIP5, or pIP7.

3.7 Screening of Clones for L. monocytogenes-specific DNA Probe

Early efforts to perform colony hybridization directly on HGMFs yielded poor results due to the very low DNA binding capacity of the polysulfone polymer on which the HGMFs are printed (Peterkin and Sharpe, 1984). The binding of denatured DNA to the surface of the membrane was improved by treating the replicated and incubated HGMF with polyethyleneimine, a polycation, prior to cell lysis (P. Entis, pers. comm.). Microwave radiation under alkaline conditions was used

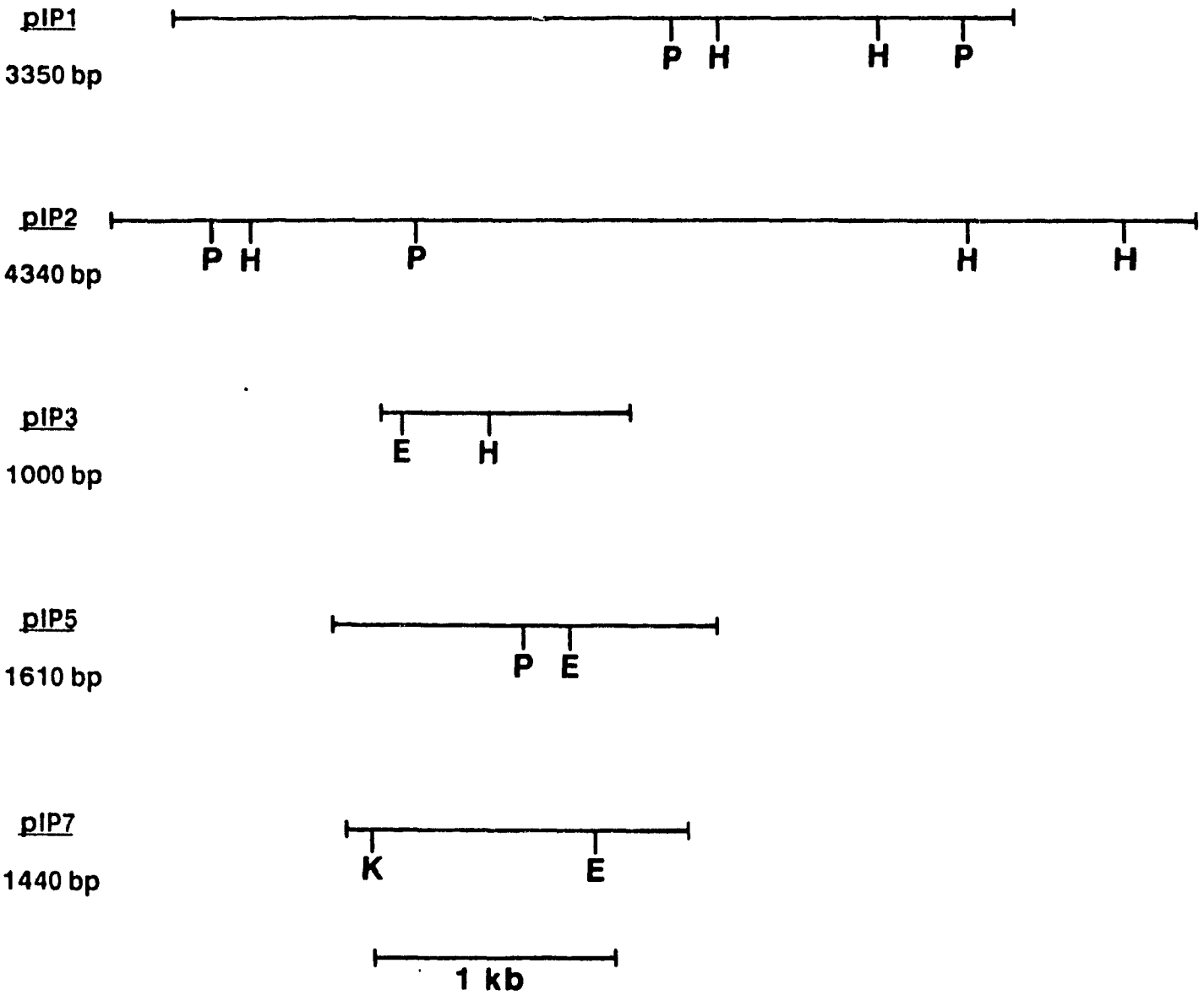

Fig. 3-8. Restriction maps of inserts from plasmids of recombinant clones showing β-hemolytic activity upon retransformation. H-Hind III; P-Pvu II; E-EcoR V; K-Kpn I.

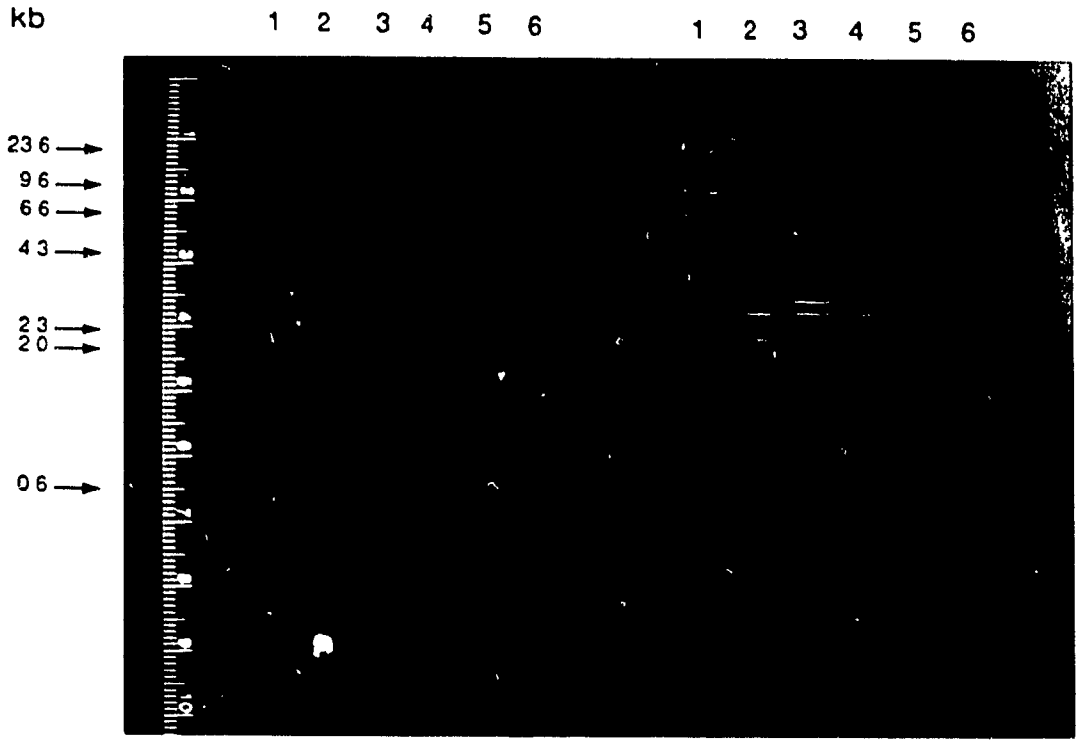

Fig. 3-9. Hind III/Kpn I double digests of plasmid DNA of hemolytic recombinant clones. Lanes 2-6: pIP1, pIP2, pIP3, pIP5, and pIP7 DNA (the linearized pUC18 plasmid vector can be seen as a 2700 bp band; lane 1: molecular size markers of λ phage DNA Hind III fragments.

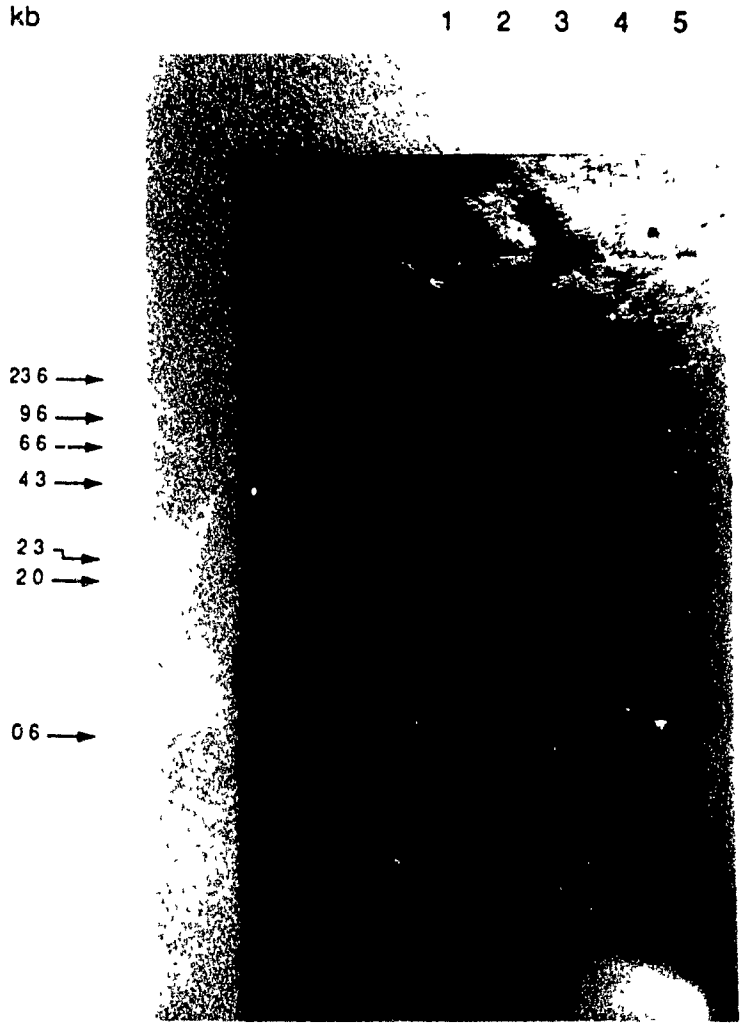

Fig. 3-10. Hybridization of pIP1 DNA to Southern blots of plasmid DNA of hemolytic recombinant clones. The Hind III/Kpn I double digests of plasmid DNA were hybridized to digoxigenin-labelled pIP1 DNA and the chromogen was visualized. Lanes 1-5: Hind III/Kpn I double digests of pIP1, pIP2, pIP3, pIP5, and pIP7 DNA.

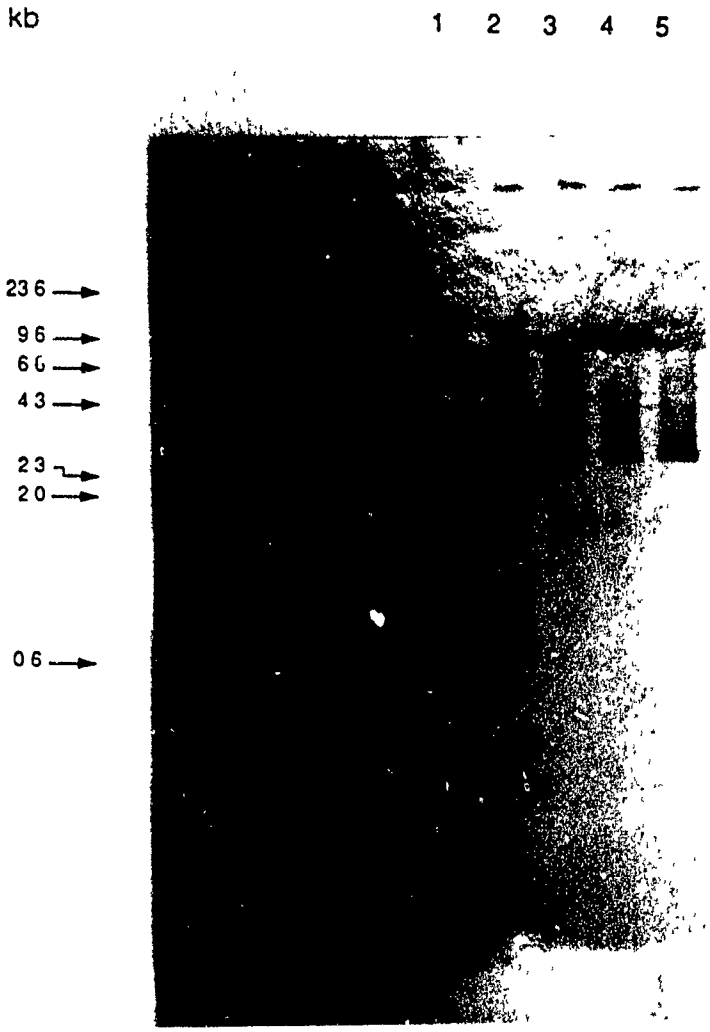

Fig. 3-11. Hybridization of pIP2 DNA to Southern blots of plasmid DNA of hemolytic recombinant clones. The Hind III/Kpn I double digests of plasmid DNA were hybridized to digoxigenin-labelled pIP2 DNA and the chromogen was visualized. Lanes 1-5: Hind III/Kpn I double digests of pIP1, pIP2, pIP3, pIP5, and pIP7 DNA.

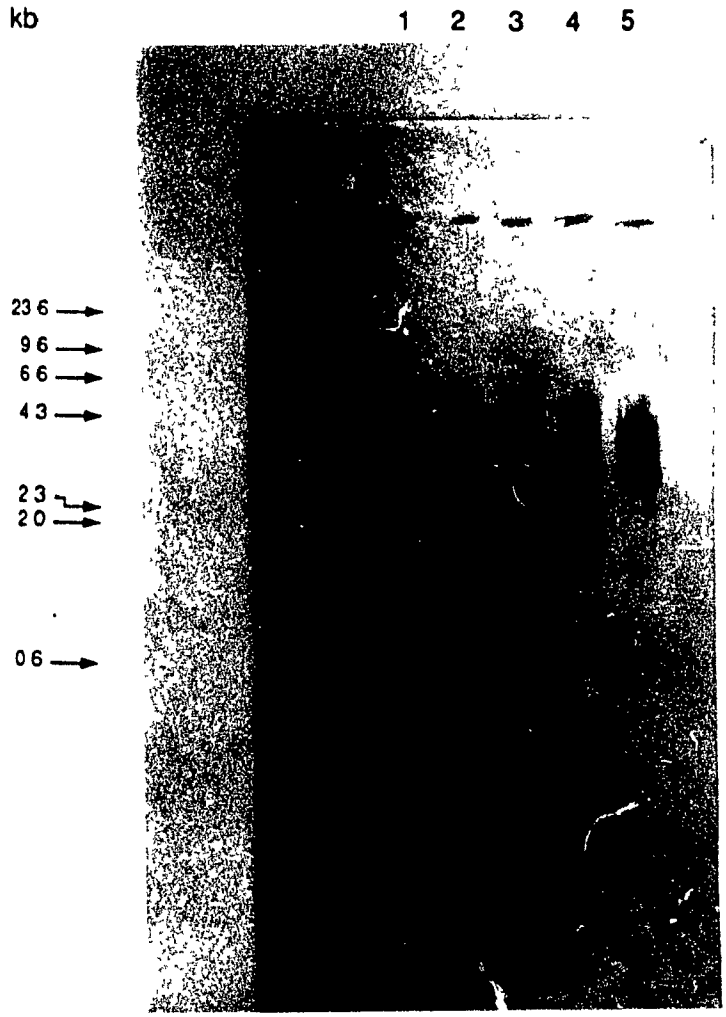

Fig. 3-12. Hybridization of pIP5 DNA to Southern blots of plasmid DNA of hemolytic recombinant clones. The Hind III/Kpn I double digests of plasmid DNA were hybridized to digoxigenin-labelled pIP5 DNA and the chromogen was visualized. Lanes 1-5: Hind III/Kpn I double digests of pIP1, pIP2, pIP3, pIP5, and pIP7 DNA.

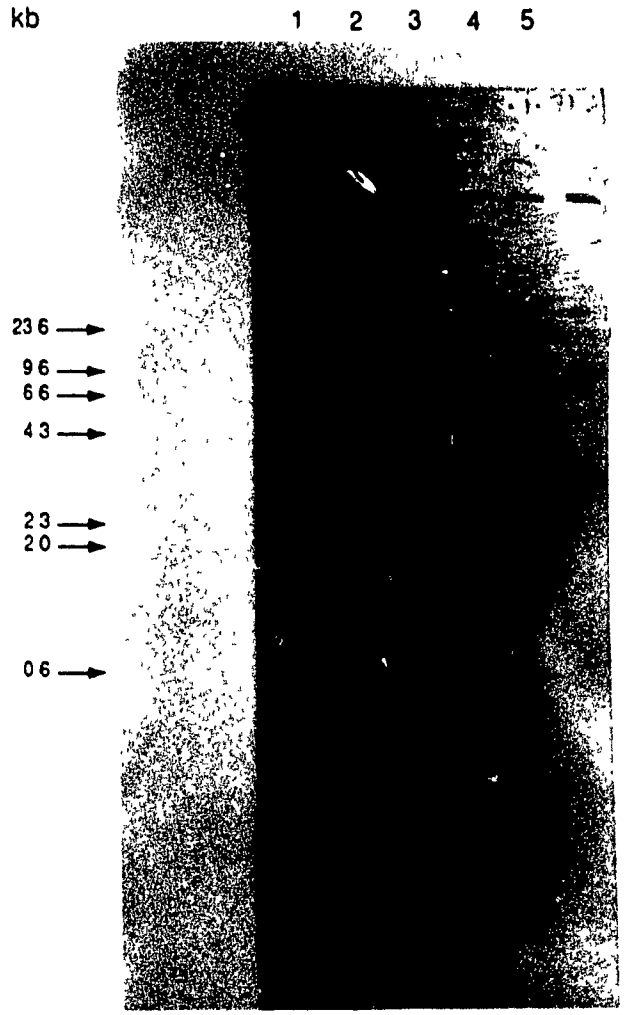

Fig. 3-13. Hybridization of pIP7 DNA to Southern blots of plasmid DNA of hemolytic recombinant clones. The Hind III/Kpn I double digests of plasmid were hybridized to digoxigenin-labelled pIP7 DNA and the chromogen was visualized. Lanes 1-5: Hind III/Kpn I double digests of pIP1, pIP2, pIP3, pIP5, and pIP7 DNA.

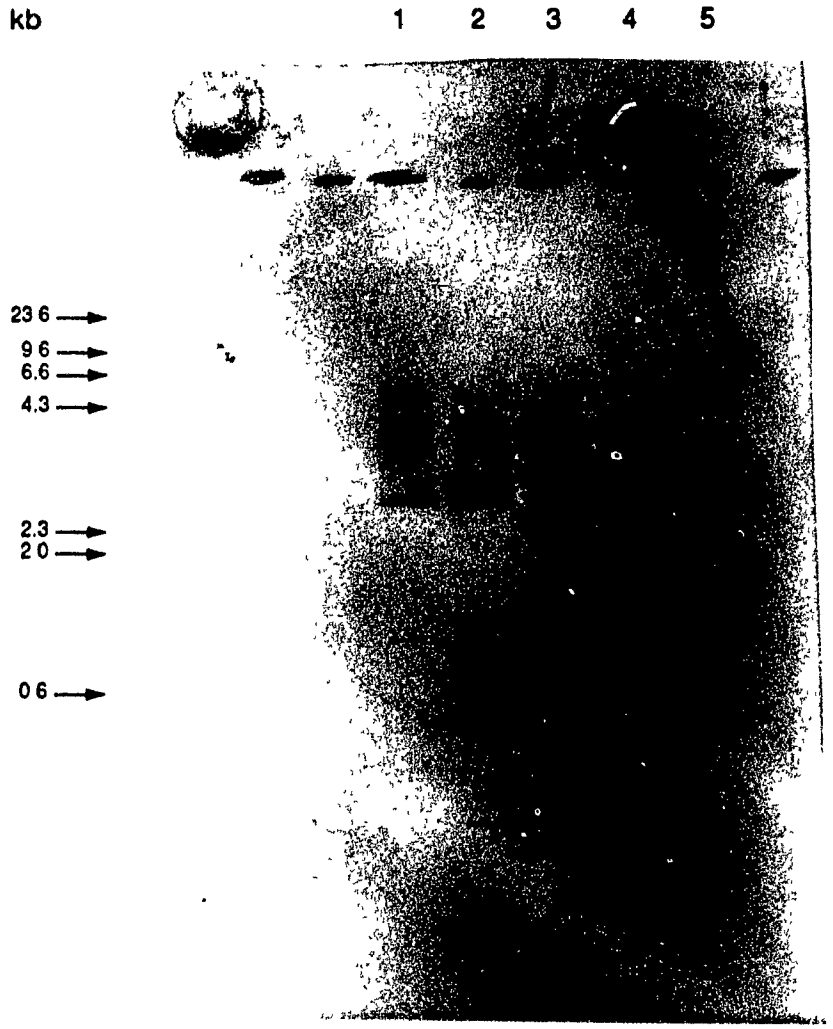

Fig. 3-14. Hybridization of pAD319 DNA to Southern blots of plasmid DNA of hemolytic recombinant clones. The Hind III/Kpn I double digests of plasmid DNA were hybridized to digoxigenin-labelled pAD319 DNA and the chromogen was visualized. Lanes 1-5: Hind III/Kpn I double digests of pIP1, pIP2, pIP3, pIP5, and pIP7 DNA.

to lyse the Gram-positive cell wall of L.monocytogenes and denature the cell DNA (Datta et al., 1987). E. coli 16S and 23S RNA hybridized to the lysed cells, demonstrating that lysis occurred (results not shown). The splotchy appearance of early autoradiographs, probably due to non-specific binding, was improved by adding a proteolytic digest step following cell lysis and DNA denaturation. This yielded a technically acceptable result (Fig. 3-15). Proteinase K was chosen for this digestion as it is free from nuclease activity. The denatured DNA was fixed to the polymer surface by UV irradiation.

For initial screening of the clones for a species-specific DNA probe, forty organisms were arrayed on a master HGMF (PROBESCREEN40; Sect. 2-11), which was replicated and incubated as required (Sharpe et al., 1989). Plasmid DNAs pIP1, pIP2, pIP3, pIP5, and pIP7 were each ^{32}P-labelled and used as probes in screening by direct colony hybridization on HGMF against these organisms (Peterkin et al., 1989c). The specificity of detection by direct colony hybridization on HGMFs was determined in comparison with colony blot hybridization on cellulose membranes against the same organisms (Table 3-6). There was no evidence of a significant difference (α = 0.05) between the results for these two methods of colony hybridization. None of the 5 recombinant plasmids, except the DNA of pIP3, hybridized with the 20 organisms of genera other than Listeria. The DNA of pIP5 demonstrated good specificity for L. monocytogenes, showing sequence homology with the 10 L. monocytogenes strains whilst hybridizing with none of the other Listeria spp., except for some weak homology with one strain of L. innocua in colony blots, though not in direct colony hybridization on HGMF.

To expand the screening by the inclusion of other L. monocytogenes strains, the master HGMF of PROBESCREEN100 (Sect. 2-11) was inoculated. As required, it was replicated and incubated as before (Fig. 3-16). ^{32}P-labelled DNA of plasmids pIP1, pIP2, pIP3, pIP5 and pIP7 were tested by direct colony hybridization on HGMFs against this array of organisms. Only the pIP5 probe did not hybridize with other Listeria spp. whilst showing sequence homology with all 70 L. monocytogenes strains tested (Fig. 3-17).

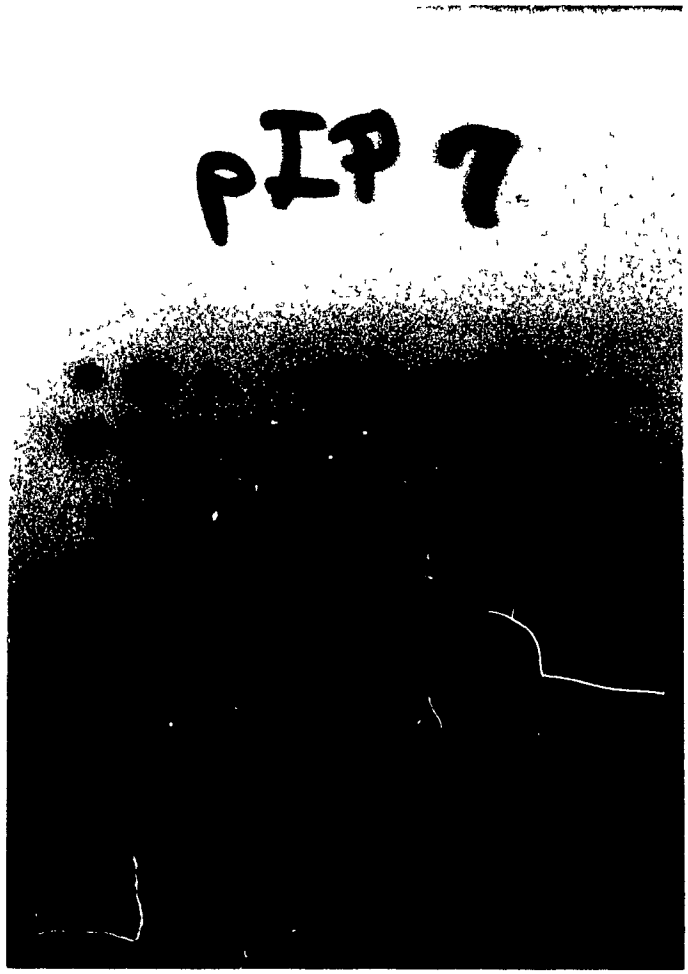

Fig. 3-15. Autoradiogram of hybridization on HGMF (^{32}P-labelled pIP7 DNA against 40 organisms). For a direct colony hybridization on HGMF, a proteinase K digestion step was added after the cells were lysed and the DNA was denatured by microwave irradiation. The autoradiograms obtained were of good quality, without the high background which had previously marred the procedure.

Table 3-6. Detection of L. monocytogenes using DNA probes - comparison of hybridization to colony blots and direct colony hybridization on HGMF[a]

Organism	pIP1		pIP2		pIP3		pIP5		pIP7	
	CB[b]	HGMF[c]	CB	HGMF	CB	HGMF	CB	HGMF	CB	HGMF
1.L. monocytogenes 1c	+	+	+	+	+	+	+	+	+	+
2.L. monocytogenes 1/2	+	+	+	+	+	+	+	+	+	+
3.L. monocytogenes 1/2c	+	+	+	+	+	+	+	+	+	+
4.L. monocytogenes 3	+	+	+	+	+	+	+	+	+	+
5 L. monocytogenes 4	+	+	+	+	+	+	+	+	+	+
6.L. monocytogenes 4b	+	+	+	+	+	+	+	+	+	+
7.L. monocytogenes 4c	+	+	+	+	+	+	+	+	+	+
8.L. monocytogenes 4d	+	+	+	+	+	+	+	+	+	+
9 L monocytogenes 4e	+	+	+	+	+	+	-	+	-	+
10.L monocytogenes 4ab	$+_w$[d]	$+_w$	+	+	+	+	+	+	+	-
11.L innocua	$+_w$	$+_w$	+	+	+	+	$+_w$	-	+	+
12.L. innocua	$+_w$	$+_w$	+	$+_w$	+	+	-	-	+	+
13.L. welshimeri	-	-	+	+	+	+	-	-	+	+
14.L seeligeri	+	+	+	+	+	+	-	-	+	+
15 L seeligeri	-	-	-	-	+	+	-	-	+	+
16.L. ivanovii	-	-	-	-	+	+	-	-	+	+
17.L ivanovii	-	-	-	-	+	+	-	-	+	+
18.L. grayi	-	-	-	-	+	+	-	-	-	-
19 L. murrayi	-	-	-	-	+	+	-	-	-	-
20 L. denitrificans	-	-	-	-	+	-	-	-	-	-
21.E rhusiopathiae	-	-	-	-	-	-	-	-	-	-
22.L casei	-	-	-	-	-	-	-	-	-	-
23 B thermosphacta	-	-	$+_w$	$+_w$	+	+	-	-	-	-
24.C. aquaticum	-	-	-	-	+	+	-	-	-	-
25 A haemolyticum	-	-	-	-	+	+	-	-	-	
26.Oerskovia spp.	-	-	-	-	-	-	-	-	-	-
27.K zopfii	-	-	-	-	-	-	-	-	-	-
28 E. coli	-	-	-	-	+	+	-	-	-	-
29 C. freundii	-	-	-	-	+	+	-	-	-	-
30.K. pneumoniae	-	-	-	-	+	+	-	-	-	-
31.S. johannesburg	-	-	-	-	+	+	-	-	-	-
32.E aerogenes	-	-	-	-	+	+	-	-	-	-
33.P. aeruginosa	-	-	-	-	+	+	-	-	-	-
34.A hydrophila	-	-	-	-	+	+	-	-	-	-
35.S aureus	-	-	-	-	+	+	-	-	-	-
36 B cereus	-	-	-	-	+	+	-	-	-	-
37.Y. enterocolitica	-	-	-	-	+	+	-	-	-	-
38.E. coli	-	-	-	-	+	+	-	-	-	-
39.V vulnificus	-	-	-	-	+	+	-	-	-	-
40 G. haemolysans	-	-	-	-	-	+	-	-	-	-

[a] Probes were labelled with ^{32}P.

[b] CB - hybridization to colony blots on Whatman 541 paper of colonies grown on TSA plates.

[c] HGMF - direct hybridization to colonies grown on HGMF.

[d] $+_w$ - weakly positive.

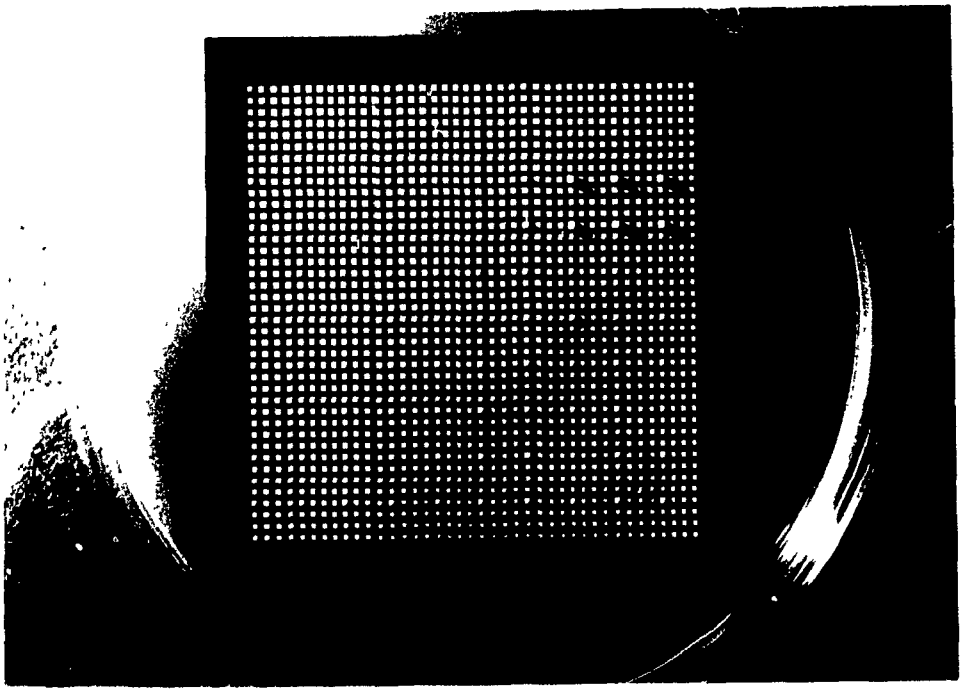

Fig. 3-16. Appearance of array of PROBESCREEN100 organisms on HGMF. Each 4th row and column are inoculated in order across and down the HGMF with the organisms listed in Tables 2-1, 2-2, and 2-3, starting with the top left-hand grid cell. After a 24 h incubation at 30°C, there is growth in all of the inoculated grid cells, but some growth which is by nature slight, such as that of the Listeria spp., does not show well in the photograph.

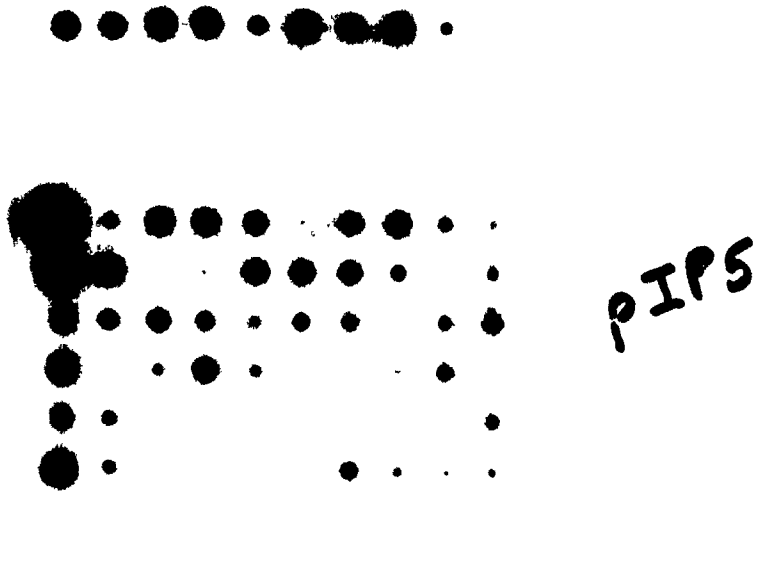

Fig. 3-17. Autoradiogram of hybridization of pIP5 DNA to PROBESCREEN100 organisms. Only the 70 L. monocytogenes strains (Rows 1, 17, 21, 25, 29, 33, and 37) show sequence homology with the ^{32}P-labelled DNA probe.

3.8 Chromogen-labelled L. monocytogenes Probe on HGMFs

Chromogen-labelled DNA probe. When HRP-labelled pIP5 plasmid DNA was screened by direct colony hybridization against the organisms of PROBESCREEN100, sequence homology was again demonstrated with only the 70 L. monocytogenes strains, and not with other Listeria spp. nor with organisms of other genera (Fig. 3-18). A 1.4 kb fragment prepared from a Kpn I/Pst I double digest of pIP5 DNA, labelled with HRP, and tested by direct colony hybridization on HGMF against the organisms of PROBESCREEN100, gave the same results. When screened in the same way, linearized pUC18 vector DNA did not show sequence homology with any of the organisms on the HGMF (results not shown). The positive purple grid-cells on the HGMFs could be easily and accurately read in the HGMF Interpreter; in the case of the HGMF shown in Fig. 3-18 the Interpreter yielded a count of 70 ± 1.1 (Peterkin et al., 1991).

3.9 Detection of L. monocytogenes in Foods by the HGMF-based DNA Probe Method

The presence or absence of L. monocytogenes in three artificially-inoculated food products was determined by either the conventional method for that food, or by the newly-developed HGMF-based L. monocytogenes DNA probe method using pIP5 DNA (Table 3-7). There was no evidence of a significant difference ($\alpha = 0.05$) between the results for these two methods of detecting the presence of L. monocytogenes in inoculated foods.

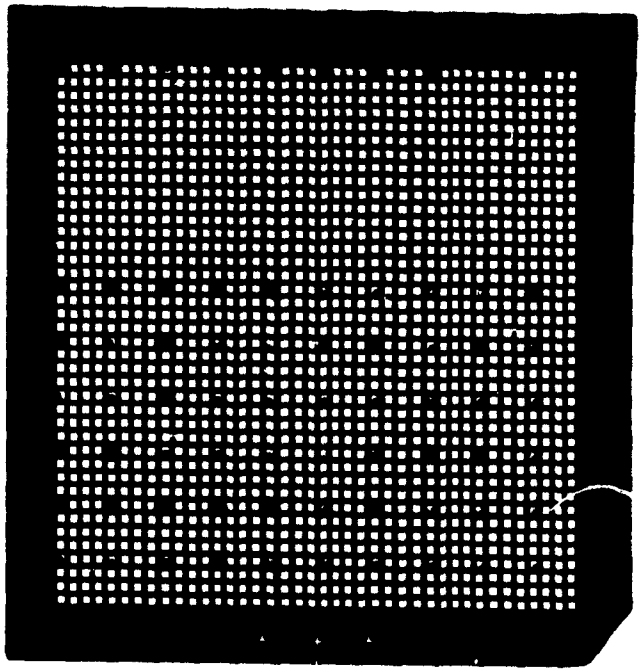

Fig. 3-18. Hybridization of the HRP-labelled pIP5 DNA probe to PROBESCREEN100 organisms on HGMF. The 70 L. monocytogenes strains show homology with the HRP-labelled DNA probe, after colour development with the substrate.

Table 3-7. Detection on HGMF of L. monocytogenes in artificially-contaminated foods - comparison of enzyme-labelled DNA probe and HPB methods[a].

Food type	Listeria spp.	No. of samples tested	Number of positive samples			
			HGMF-based DNA probe		HPB[b]	
			Low[c]	High[d]	Low	High
Soft cheese	L. monocytogenes[e]	5	5	5	5	5
	L. innocua	5	0	0	0	0
	L. welshimeri	5	0	0	0	0
	L. seeligeri	5	0	0	0	0
Raw milk	L. monocytogenes	5	4	5	5	5
	L. innocua	5	0	0	0	0
	L. welshimeri	5	0	0	0	0
	L. seeligeri	5	0	0	0	0
Ground chicken	L. monocytogenes	5	5	5	4	5
	L. innocua	5	0	0	0	0
	L. welshimeri	5	0	0	0	0
	L. seeligeri	5	0	0	0	0

[a] All uninoculated samples were negative for Listeria spp.
[b] Sensitivity of HPB method ≈ 0.2 cells. g^{-1} food.
[c] Low inoculation level - 1 cfu.g^{-1} food.
[d] High inoculation level - 100 cfu.g^{-1} food.
[e] Bacterial strains used were: L. monocytogenes 86-861, L. innocua LA-1, L. welshimeri 160H1-3, L. seeligeri 472595μA.

4. DISCUSSION

Twenty-five strains (20%) of the 122 L. monocytogenes strains examined in the course of these studies contained native plasmids. This level of incidence agrees with the findings of other researchers such as Perez-Diaz et al. (1982). Many of the plasmids isolated were very large, up to 100 kb in size. Multiple plasmids were not observed in these strains No correlation was found between virulence of the organism, as shown by the presence of ß-hemolytic activity, and the presence of a plasmid. The use of a known plasmid curing agent, acriflavine, in the primary isolation medium may lead to a loss of plasmids, so this may not be a true picture of the incidence of native plasmids in this organism. However, the low level actually found, and the lack of uniformity among the plasmid sizes, leads us to agree with other observers (Fistrovici and Collins-Thompson, 1990; Pritchard and Donnelly, 1990) that plasmid profiles are of little practical value in the strain typing of L. monocytogenes. Of 11 strains of other Listeria species examined, native plasmids were found in 7. Both L. ivanovii strains examined carried a 71 kb plasmid, indicating the possibility of their being the same strain However, the number of strains of other Listeria species examined was too small to be able to draw final conclusions

The genomic library (GL4) of L monocytogenes 81-861 (serovar 4b), prepared in pUC18 and used to transform E. coli DH5α host cells, showed expression of ß-hemolytic activity in 7 of its clones, as tested by the development of zones of clearing surrounding the clones on horse blood agar. However, retransformation of E. coli cells with the isolated DNA from these 7 plasmids, showed that the hemolytic activity was expressed stably in only 5 of the clones This loss of ß-hemolytic activity upon retransformation could be explained in one of the following ways: a) originally, a mixed culture of ß-hemolytic and non-hemolytic clones existed in the colony showing insertional inactivation with, eventually, the non-hemolytic clone being selected, or b) lack of expression of the ß-hemolysin determinant, or lack of extracellular secretion of the ß-hemolysin The first possibility is not likely, as only 1 recombinant plasmid was purified from each of the clones which did not show ß-hemolytic activity upon retransformation. Non-expression of the

hemolytic determinant or intracellular retention of the ß-hemolysin are possible explanations for the loss of extracellular hemolytic activity upon retransformation. Studies comparing the intracellular proteins and the insert DNAs of those clones which retained, and those which lost, their ß-hemolytic activity could be used to elucidate the fate of the hemolysin, or to study the regulation mechanisms of the hemolysin determinant.

As the ß-hemolysin, listeriolysin O, is strongly implicated in the pathogenicity of this organism (Cossart and Mengaud, 1989), the 5 hemolytic clones were considered likely candidates as a source of DNA for a species-specific probe for L. monocytogenes. It was also thought to be of interest to examine these clones for possible virulence, as their inserts contained a ß-hemolysin determinant which might be separate from other virulence factor determinants. We also considered that these clones were a source of material for establishing whether other ß-hemolysin determinants apart from hlyA may exist in this strain of L. monocytogenes.

The 5 ß-hemolytic clones, representing 0.2% of the L. monocytogenes gene bank, contained recombinant plasmids (pIP1, pIP2, pIP3, pIP5, and pIP7) which were stably maintained in the host cells, and a hemolytic activity that was stably expressed. The first reported L. monocytogenes (serovar 1/2) genomic library contained chromosomal fragments of about 35 kb size packaged in a cosmid vector, and expressed in E. coli HB101 (Vicente et al., 1985). Twelve ß-hemolytic clones were found by these workers during screening of 2000 clones (0.6%) but, in contrast to the genomic library reported here, the recombinant plasmids were unstably maintained. Leimeister-Wächter and Chakraborty (1989) prepared an L. monocytogenes (serovar 1/2a) gene bank of 8 kb fragments in a plasmid vector, expressed in E. coli DH5α. In screening 10,000 recombinants of this gene bank ß-hemolytic activity was expressed in only 2 weakly-hemolytic clones (0.02%). The product causing the hemolysis was not cross-reactive with anti-listeriolysin O antibodies. Though the products of some of their other clones did react with the antibodies, these latter recombinants did not demonstrate hemolytic activity in the microtitre plate

assay, even after 3 d of incubation. This contrasts with the ß-hemolysis of our clones which showed clearly on horse blood agar plates after overnight incubation.

The virulence of our clones was tested by intraperitoneal injection into mice. The 5 clones which expressed ß-hemolytic activity, caused the death of from 2/5 to 5/5 mice, in duplicate experiments. The 4 types of negative controls, consisting of cells of L. innocua, of the host cell strain, of the host cell strain containing pUC18, or of the host cell strain containing a recombinant plasmid which did not confer hemolytic activity to the cell, caused neither illness nor death over the experimental period, when administered to mice under the same experimental conditions. The virulence was therefore not due to the host strain, the plasmid vector, nor to non-hemolytic L. monocytogenes inserts. The only virulent strains, apart from the L. monocytogenes positive control, were the recombinants possessing a strong, stably expressed ß-hemolytic activity This indicates that the addition of an L. monocytogenes ß-hemolytic activity to a non-pathogenic bacterium such as the host E. coli cell may be sufficient to cause a lethal effect (Peterkin et al., 1990). However, there are as yet insufficient data to identify the proteins possessing β-hemolytic activity as being responsible for mouse pathogenicity. Virulence determinants other than those for a β-hemolysin may also be encoded by the cloned DNA These results can be compared to those presented in a recent report of the cloning of hlyA into Bacillus subtilis yielding a hemolytic phenotype (Bielecki et al., 1990). After being internalized by a phagocytic macrophage-like cell line, the hemolytic B. subtilis grew within the macrophage cytoplasm, showing that a single gene product is sufficient to convert a non-pathogenic bacterium into an intracellular parasite (Bielecki et al., 1990).

The results of the CAMP tests of the hemolytic recombinant clones support the current understanding that the CAMP factor is a protein separate from the ß-hemolysin (Kreft et al., 1989). The ß-hemolysin, listeriolysin O, has been shown to be required for virulence in L. monocytogenes (Cossart and Mengaud, 1989). However, the ß-hemolytic clones of this study showed lethality towards mice whether CAMP factor activity was present or not. We conclude that in our hemolytic

recombinant clones there was no correlation shown between the presence of the CAMP factor and virulence in mice.

Measurement of the hemolytic activity of broth cultures of the clones by the microtitre plate assay showed varying levels ranging from 1 to ≥8 units, compared to an activity of ≥8 units for the L. monocytogenes 81-861 control. However, hemolytic activity was not found in supernatants of broth cultures, nor in soluble, nor membrane-associated, cell fractions prepared by disrupting the cells by sonication. Vicente et al. (1987) demonstrated hemolytic activity in the supernatants from sonicated cells of hemolytic clones. Leimeister-Wächter and Chakraborty (1989) reported on E. coli recombinant clones containing L. monocytogenes genomic DNA which they identified by Western blotting using anti-listeriolysin O antisera. The authors found no zones of hemolysis when these clones were grown on blood agar plates. However, when cells grown at 30°C were disrupted by lysozyme digestion followed by freezing and thawing, a hemolytic activity was demonstrated in the microtitre plate assay. We were unable to demonstrate the hemolytic activity of our clones in this way. At present, there is no explanation of the discrepancy between the results obtained by these workers and ourselves. Though we have not yet been able to demonstrate the hemolytic activity of these recombinant clones dissociated from whole cells, the hemolysin must be released outside the cells, else it could not form a zone of clearing surrounding the colonies on horse blood agar plates. The hemolytic activity of these clones is cell-associated but not intracellular. As the ß-hemolysin, listeriolysin O, is a thiol-dependent hemolysin (Geoffroy et al., 1989), the addition of 6 mM cysteine to all of these fractions was tested. This treatment had no effect on the results, indicating that the absence of demonstrated ß-hemolytic activity in cell-free fractions was not due to oxidation of thiol groups.

The cytolytic activity of membrane-filtered supernatants of L. monocytogenes broth cultures against CHO cells has been shown previously to correlate well with the hemolytic activity of the same preparation (Farber and Speirs, 1987a). The slight cytolytic activity against CHO cells shown by the filtered supernatants in which we were unable to demonstrate hemolytic activity,

may indicate that this test has a greater sensitivity than the hemolysin microtitre assay. The hemolytic/cytolytic activity may therefore be present in the various broken cell fractions, but not in sufficient concentration to give a positive result in the microtitre plate assay against sheep red blood cells.

From the first identification of the ß-hemolytic clones during the screening of the 2500 recombinant clones of the L. monocytogenes 81-861 gene bank, 1 plasmid only has been isolated from each. These plasmids have been stable, in that they maintained the same molecular size over the course of these studies. The recombinant plasmids of the 5 hemolytic clones showing stable ß-hemolytic activity had inserts ranging from 1000 to 4340 bp. Preliminary restriction mapping of the inserts from these ß-hemolytic clones showed that two of the recombinant plasmids, pIP1 and pIP2, possessed a 650-bp internal HindIII fragment, characteristic of the hlyA determinant of listeriolysin O (Mengaud et al., 1988b). This suggested that the L. monocytogenes DNA inserts in these plasmids might contain the listeriolysin O gene. The shorter inserts of pIP3, pIP5, and pIP7, however, showed a pattern of DNA restriction sites different from that of pIP1 and pIP2. They possessed an EcoR V site not seen in the 2 larger inserts, and might share homologous sequences.

To examine the homology of these inserts, Southern blots of Hind III/Kpn I double digests of each of the recombinant plasmids were hybridized to digoxigenin-labelled pIP1, pIP2, pIP5, and pIP7, with the results shown in Figs. 3-10 to 3-13. Apart from hybridization to the common 2.7-kb band of the pUC18 vector DNA, no homology was shown between the DNA of these 5 recombinant plasmids. This unexpected result was confirmed in a repeat experiment. These hybridization experiments were performed under conditions of high stringency.

A more complete restriction map of the inserts was now developed (Fig. 3-8), and the results were in keeping with the results of the probing of the Southern blots. The insert of pIP1 contained a Pvu II site 220 bp upstream from the Hind III fragment, that was missing in the pIP2 insert. Also, no Pvu II site exists at this position in the published sequence of hlyA (Mengaud et

al., 1988). This suggested that despite both possessing a 650-bp Hind III fragment, the inserts of pIP1 and pIP2 were different from one another. Though the inserts of pIP3, pIP5, and pIP7 each possessed an EcoR V site, as described above, restriction sites of other enzymes indicated a lack of common sequences. The pIP5 insert with its Pvu II site 190 bp upstream of, and its lack of a Hind III site 360 bp downstream of, the EcoR V site, lacked additional restriction sites shared with pIP3 and pIP7. The inserts of pIP3 and pIP7 also seemed not to share restriction sites. This latter conclusion depended on the accuracy of the size determinations of the 360-bp internal EcoR V/Hind III fragment of pIP3 insert, and of the 390-bp length of pIP7, from the EcoR V site to the 3'-terminus.

The premise that the DNA sequences of the inserts were different from each other was reinforced by the results of the colony hybridizations when these recombinant plasmids were tested against a battery of 20 Listeria strains and strains of 20 other species as shown in Table 3-6. Though pIP1 and pIP2 showed similar homologies, pIP2 hybridized to a Lactobacillus sp., whereas, pIP1 did not. The hybridization patterns of the remaining probes were different from each other, and from those of pIP1 and pIP2. The pIP3 probe showed homology with all of the Listeria species and, surprisingly, with 16 out of the 20 organisms of other species. The pIP5 probe was specific only for L. monocytogenes, with the exception of a weak hybridization to an L. innocua strain in the colony blot, but not the HGMF, procedure. The pIP7 probe showed essentially the same homology with Listeria species as did pIP3, but no homology with the 20 other species.

We then wished to demonstrate which inserts might contain the L. monocytogenes hlyA gene. Southern blots of Hind III/Kpn I double digests of each of the recombinant plasmids were hybridized to digoxigenin-labelled pAD319, containing the hlyA gene cloned in pUC8 (Datta et al., 1990). Hydridization was seen with the common 2.7-kb band of the pUC18 vector DNA, indicating the homology that exists between the 2 cloning vectors, pUC18 and pUC8. In addition to this, homology was demonstrated only between pAD319 and pIP2, with hybridization being seen with

the 3000, 650, and 300 bp bands of the pIP2 double digest. There was no homology evident with the inserts of the remaining plasmids. We conclude that the hlyA gene has been cloned in pIP2, but not in the other plasmids of this study.

We have presented genetic evidence for the existence of two or more hemolysin determinants apart from hlyA in L. monocytogenes 81-861. The expression products of these genes may also be virulence factors. It is of interest that a ß-hemolysin apart from listeriolysin O has now been reported in serovars 1/2 (Vicente et al., 1987a), 1/2a (Leimeister-Wächter et al., 1987), and 4b (this report) of the organism.

There have been several reports that a second hemolysin, immunologically distinct from listeriolysin O, may be present in some L. monocytogenes strains (Chakraborty et al., 1986; Leimeister-Wächter et al., 1987; Vicente et al., 1987a). This hemolytic activity was first described by Parrisius et al. (1986), who screened 28 ß-hemolytic strains for the presence of listeriolysin with an immunoblot assay. Only 2 of these strains produced a streptolysin O-related hemolysin, the remaining ß-hemolytic strains being negative in the immunoassay. The authors proposed that the streptolysin o-related toxin be named α-listeriolysin (now known as listeriolysin O), and that the remaining hemolysin(s) be termed ß-hemolysin. In addition, two types of hemolysin were identified in clones from a L. monocytogenes gene bank constructed in E. coli (Chakraborty et al., 1986; Goebel et al., 1988). The first was a 23,000 Da protein, possibly the CAMP factor, which was not SH-activated, and did not cross-react with anti-listeriolysin nor anti-SLO antibodies. The other cross-reacted with anti-SLO, but activation by SH groups was not tested (Leimeister-Wachter et al., 1987). Vicente et al. (1987a) identified 12 recombinants expressing ß-hemolytic activity after the cloning of L. monocytogenes genomic DNA into E. coli host cells. Subcloning of Hind III restriction fragments of the plasmid from one of these clones resulted in the preparation of a stable hemolytic clone with an 8.3 kb insert. Further subcloning was used to prepare clones whose hemolytic activity was only detectable after sonication. Gel filtration of the sonicated preparation led to the elution of two peaks of hemolytic activity, corresponding to proteins of 22

kDa and 48 kDa, suggesting the existence of two hemolysins. L. ivanovii also secretes 2 cytolytic factors - one is a thiol-activated hemolysin of 61 kDa, termed ivanolysin O, and the other a 27 kDa sphingomyelinase C that may be involved in the synergistically-enhanced hemolysis, in other words, CAMP factor activity (Vazquez-Boland et al., 1989).

One explanation of the results obtained in this study is that there are 5 genetically-distinct proteins possessing ß-hemolytic activity in this strain of L. monocytogenes. The other possibility is that these 5 inserts may represent the determinants for separate and truncated sections of fewer than 5 ß-hemolysins, with these truncated sections not only being hemolytic but also virulent. As separate centres of activity rarely exist within a protein molecule, we think that this latter is not a probable explanation.

Though it may be difficult to conceive of 5 distinct membrane-active proteins in one organism, this situation is not without precedent in another Gram-positive bacterium. Bacillus cereus elaborates a variety of extracellular membrane-active enzymes and cytolytic toxins (Gilmore et al., 1989). These include a 1) phospholipase C (lecithinase), 2) sphingomyelinase, 3) phosphatidylinositol phospholipase C, 4) cereolysin, a thiol-activated hemolysin in the same class as listeriolysin O, and 5) a heat-stable cytolysin about which little is known. The sphingomyelinase C is similar to the S. aureus CAMP factor, and acts synergistically with the phospholipase C or the phosphatidylinositol phospholipase C (acting as the Streptococcus agalactiae CAMP factor), to enhance hemolysis. In addition, Mengaud et al. (1991) recently examined the role of lecithinase in virulence using transposon-induced lecithinase-negative mutants of L.monocytogenes. These mutants possessed reduced virulence. Also, transposon mutants of L. monocytogenes lacking a phosphatidylinositol-specific phospholipase C have recently been reported to be avirulent for mice, and to be defective for intracellular growth (Camilli et al., 1991). Both authors reported that these transposon mutants were normal for hemolysin production. Therefore it seems that there may be a role for several membrane-active proteins amongst bacterial virulence factors.

It is also possible that a regulatory gene has been cloned which activates the transcription of a β-hemolytic determinant in the E. coli genome. Such a situation has been reported by Soltes and MacInnes (1991) who demonstrated significant DNA sequence homology between a cloned gene (named hly X because it conferred a β-hemolytic phenotype) of Actinobacillus pleuropneumoniae, and the fnr gene of E. coli.

The recombinant clones of the present study may furnish material towards elucidating the gene sequences and clarifying the various types of membrane-active or regulatory proteins that may exist in this strain of L. monocytogenes. At present, however, as the cell-free proteins have not been available for study, nothing is known about their molecular weight or activities.

Several radio-labelled DNA probes for the detection of either Listeria spp. or L. monocytogenes specifically, have been reported (Datta et al., 1987; Chenevert et al., 1989). Notermans et al. (1989) developed a radio-labelled DNA probe encoding a delayed hypersensitivity factor which detected L. monocytogenes and L. ivanovii strains in a colony hybridization method. The only probe reported for food use with a colorimetric label is the commercial probe sold by Gene-Trak Systems (Klinger and Johnson, 1988). However, this has the disadvantage of being genus- rather than species-specific. As there is no evidence of other species in the Listeria genus than L. monocytogenes being pathogenic to humans (Farber and Losos, 1988), it could be argued that it is preferable to use a DNA probe in foods to detect the pathogenic species, rather than using members of the genus Listeria as indicator organisms.

The pIP5 DNA probe for L. monocytogenes developed in the course of this work showed excellent specificity for this species only, and did not react with other species in the genus Listeria (Peterkin and Idziak, 1986; Peterkin et al., 1991). This was tested with 70 L. monocytogenes strains in pure culture, including all serovars. It did not hybridize with a wide selection of related genera, including Erysipelothrix, Brochothrix, and Lactobacillus, nor with other foodborne pathogens such as Salmonella spp., S. aureus and Yersinia spp. Furthermore, the probe was successfully conjugated to a chromogen, horseradish peroxidase, so that it was no longer

necessary to use a radioactive label which might be unacceptable in a food manufacturing environment. This is the first report of the use of a DNA probe for direct hybridization on HGMF, labelled either with a radioactive or a chromogenic label (Peterkin et al., 1989b).

The use of the chromogenic label led to the additional advantage of electronic data acquisition for detection and enumeration of L. monocytogenes with this probe. The HGMF Interpreter was able to read the positive grid-cells accurately after development of the DNA probe label, either with pure cultures of organisms arrayed on an HGMF, or with inoculated foods. The validity of automated counting by the HGMF Interpreter has previously been compared with manual counting by two analysts on 7 types of food (Sharpe and Peterkin, 1988). It showed no significant method differences (α = 0.05).

This colorimetric DNA probe method, a direct colony hybridization technique on HGMF, showed no statistical differences at a 95% significance level, from the accepted colony hybridization technique using colony blots. Furthermore, when tested in inoculated foods (soft cheese, raw milk, ground chicken) in comparison with the conventional method appropriate to that food, the method showed no significant differences (α = 0.05) in the recovery of L. monocytogenes from those foods.

The work presented here also showed an enlargement of the potential of the HGMF in general microbiology, in food microbiology and in molecular genetics. During the preparation of genomic library GL1 of L. monocytogenes 81-861 (serovar 4b), the few thousand transformants required to encompass the entire bacterial genome were inoculated by filtration on a series of 40 HGMF at a cell density of ≈60 per filter. From the relationship:

$$P = -\frac{N-X}{X} \log_e \frac{N-X}{X}$$

where P = the probability of purity of each clone, N = the total number of grid-cells (1600 for the commercial Iso-Grid) and X = the number of occupied cells, it was calculated that at this cell density, there would be a 98% probability of the growth in any one grid-cell coming from a single

clone (Peterkin et al., 1987). The clones were replicated to fresh HGMFs and grown overnight on agar containing tetracycline in order to determine the number with insertional inactivation. This accurate estimation of the probability of purity can be of assistance both in the screening of transformed clones when choosing those recombinants that will form a gene bank, and when purifying strains of organisms in general microbiology.

The HGMF also formed the basis of a strain storage system (Peterkin et al., 1989c; Sharpe et al., 1989), whereby 400 strains per HGMF can be stored for periods of 2 or more years at -70°C. These can be thawed and replicated as required. Furthermore, these libraries of up to 400 strains per HGMF form the basis of an efficient screening system when screening putative DNA probes against many organisms to test specificity and sensitivity (Peterkin et al., 1991).

5. CONCLUSIONS

1. Twenty-five (20%) of the 122 L. monocytogenes strains tested contained native plasmids with molecular sizes ranging from 2.0 to 100 kb. There was no relationship discernible between the presence of the plasmids and phenotypic characteristics such as hemolytic activity. Seven of the 11 strains of other Listeria spp. examined showing native plasmids ranging from 58 to 71 kb in size.

2. A genomic library was developed in which L. monocytogenes 81-861 hemolysins were expressed in E. coli at a rate of about 0.2% of the 2500 clones examined. The recombinant plasmids were stably maintained in the host cell, and the hemolytic activity stably expressed.

3. A L. monocytogenes 81-861 ß-hemolysin determinant may be a sufficient cause of virulence when expressed in a non-pathogenic bacterium, E. coli.

4. There is genetic evidence for the presence of one or more β-hemolysin determinants, other than the listeriolysin O gene, in L. monocytogenes 81-861.

5. A method for performing direct colony hybridization on HGMFs was developed. When using radiolabelled DNA probes, there was no evidence of a significant difference ($\alpha = 0.05$) between colony blot hybridization and the newly-developed HGMF method.

6. A radiolabelled DNA probe was shown to be specific for L. monocytogenes strains by screening against many bacterial strains on HGMFs. The specificity of the probe was also

demonstrated using a newly-developed HGMF colony hybridization method with the DNA probe labelled with horseradish peroxidase.

7. When the HGMF-based colorimetric DNA probe method was compared to a conventional method for detecting the presence of L. monocytogenes in artificially-inoculated foods, there was no evidence of a significant difference ($\alpha = 0.05$) between the results for the two methods of detection. Following a 24 h enrichment step, confirmed identification was obtained in 48 h, as compared to a 3-4 days by conventional methods.

8. L. monocytogenes was detected and counted electronically by the HGMF Interpreter, when the colorimetric DNA probe method on HGMFs was used.

9. The HGMF was, in addition to its conventional uses, used for the determination of probability of purity of bacterial cultures, and for the screening and storage of recombinant clones.

6. APPENDICES

Appendix A. Restriction fragment sizes[a] of hemolytic recombinant plasmids.

Plasmid (size in bp)	Hind III	Pvu II	EcoR V	Kpn I
pIP1 (6040)	3200 2100 650	2250 2250 960 420	no sites	5900
pIP2 (7030)	2950 2950 650 600	3400 2300 850 570	no sites	7000
pIP3 (3690)	3250 490	2300 1350	3800	3800
pIP5 (4300)	4200	2300 1000 900	4300	4360
pIP7 (4130)	4100	2400 1750	4200	3700 400

[a] sizes in bp

Appendix B. Double digests of hemolytic recombinant plasmids - calculated and experimental restriction fragment sizes[a].

Plasmid (size in bp)	Hind III/Kpn I		Hind III/Pvu II	
	Calculated	Experimental	Calculated	Experimental
pIP1 (6040)	2650	2700	2360	2250
	2100	2080	2100	2100
	650	660	650	650
	550	560	420	430
			370	360
			220	220
			100	100
pIP2 (7030)	2950	3000	2360	2300
	2650	2700	2250	2100
	650	650	700	760
	600	600	650	650
	300	300	500	600
			470	470
			150	200
			100	100
pIP3 (3690)	2650	2700	2360	2300
	600	660	800	860
	500	500	500	500
			100	100
pIP5 (4300)	2650	2700	2360	2300
	1550	1680	950	1000
			800	800
			100	100
pIP7 (4130)	2650	2700	2360	2300
	1050	1200	1600	1650
	400	350	100	100

[a] sizes in bp

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