Yersinia enterocolitica

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genen betrokken bij aanpassing aan lage temperatuur

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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The Road goes ever on and on
down from the door where it began.
Now far ahead the Road has gone
and I must follow, if I can.
Pursuing it with weary feet
untill it joins some larger way,
where many paths and errands meet.
And whither then? I cannot say...

(Bilbo and Frodo Balings, hobbits,
in: 'The Lord of the Rings',
J.R.R. Tolkien)

Opgedragen aan
mijn vader
(† 07-09-1992)

CONTENTS

PREFACE

Scope of the thesis

This thesis concerns the bacterium *Yersinia enterocolitica*, with special attention given to the molecular regulation of its ability to grow and multiplicate at temperatures around 0°C.

Y. enterocolitica is an important human entero-invasive pathogen with a global distribution [Bottone 1977, 1997; Ostroff 1995]. In contrast to other common bacterial enteropathogens, such as *Salmonella, Campylobacter* and *Shigella* species, which cease to grow below circa 8°C, *Y. enterocolitica* is able to grow near 0°C [Stern & Pierson 1979] and even at sub-zero temperatures [Bergann *et al.* 1995]. On account of this cold-tolerance, it has been frequently suggested that the ever increasing application of refrigeration in food storage plays a central role in the continuing expansion of *Y. enterocolitica* as a human pathogen [Christensen 1987; Mollaret 1995]. It could plausibly be argued that this cold-tolerant organism has taken advantage of the forthcoming of a new niche, mindful of Baas Becking's conclusion that "... *life is everywhere, but the environment determines its manifestation.*" [Baas Becking 1927]. Obviously, on the other hand, a causal connection is difficult to prove. Nevertheless, the fact that this hypothesis has never been scientifically documented makes it hard to rate the significance of bacterial cold-tolerance as an indirect threat to human health at its true value. Gaining insight into the suggested relationship requires a closer look at both the history of this bacterium and the milestones in the development of the 'Cold Chain' of food preservation.

Assuming that the ability of *Y. enterocolitica* to proliferate at 5°C or less, either in foods, water or other products, such as blood, truly constitutes a specific hazard for human health, the questions arise whether and how its growth at low temperature could possibly be suppressed. Answering this question, however, requires fundamental knowledge about the specific metabolic factors which enable this organism to multiply at refrigeration temperatures. Unfortunately, although the behaviour and physiology of *Y. enterocolitica* at low temperatures have been investigated extensively, only very few studies addressed the underlying mechanisms. In the present study an attempt is made to determine the vital link(s) in the metabolism at low temperatures, as a first step in elucidating how *Y. enterocolitica* copes with cold.

Outline of the thesis

Chapter 1 is devoted to *Y. enterocolitica* and describes its history and characteristics, as well as its importance as a human pathogen. In Chapter 2, the evolution of chilling into an indispensable factor of modern food preservation is delineated, including the rise of cold-loving ('psychrophilic') and cold-tolerant ('psychrotrophic') organisms in food spoilage and human disease. In Chapter 3, the present knowledge about the molecular aspects of bacterial adaptation to low temperature is reviewed. After these literature-based introductions to the bacterium under study, the development of the Cold Chain, and the phenomenon of microbial cold-adaptation, the Chapters 4 to 7 are based on experimental work with *Y. enterocolitica* and related species. In order to contribute to improved estimations of the prevalence and significance of *Y. enterocolitica*, a rapid and reliable detection method has been developed, which is described in Chapter 4. The central theme in the Chapters 5 and 6 is the identification of factors involved in cold-tolerance, as accomplished by mapping of specific phenotypic adaptations and by analysis of mutations that lead to disruption of the psychrotrophic phenotype. By the latter approach, it was deduced that expression of the *pnp*-gene, encoding polynucleotide phosphorylase (PNPase), is necessary for the ability of *Y. enterocolitica* to grow at low temperatures. Chapter 7 addresses the regulation of *pnp*-expression in *Y. enterocolitica* and other *Yersinia* species. Finally, an evaluation of the results described in the preceding chapters, and a discussion of the possible role of PNPase in the mechanisms that might enable growth at low temperature is the topic of Chapter 8.

References

1. **Baas Becking LGM.** 1927. Over de algemeenheid van het leven. (In Dutch). <About the generality of life.> Public lecture given on October 3rd 1927 at the University of Utrecht.
2. **Bergann T, Kleemann J, and Sohr D.** 1995. Modelluntersuchungen zur Psychrotrophie von *Yersinia enterocolitica*. *J Vet Med B* **42**: 523-531.
3. **Bottone EJ.** 1977. *Yersinia enterocolitica*: a panoramic view of a charismatic microorganism. *Crit Rev Microbiol* **5**: 211-241.
4. **Bottone EJ.** 1997. *Yersinia enterocolitica*: the charisma continues. *Clin Microbiol Rev* **10**: 257-276.
5. **Christensen SG.** 1987. The *Yersinia enterocolitica* situation in Denmark. *Contr Microbiol Immunol* **9**: 93-97.
6. **Mollaret HH.** 1995. Fifteen centuries of yersiniosis. *Contr Microbiol Immunol* **13**: 1-4.
7. **Ostroff S.** 1995. *Yersinia* as an emerging infection: epidemiologic aspects of Yersiniosis. *Contr Microbiol Immunol* **13**: 5-10.
8. **Stern NJ, and Pierson MD.** 1979. *Yersinia enterocolitica*: a review of the psychrotrophic water and foodborne pathogen. *J Food Sci* **44**: 1736-1742.

1

Yersinia enterocolitica

a versatile human enteric pathogen

ABSTRACT

Y. enterocolitica **is a Gram-negative bacterium which belongs to the family of the Enterobacteriaceae. Within this species, several bio-/serotypes are distinguished which are pathogenic to humans. Infection with virulent *Y. enterocolitica* mainly causes an acute gastroenteritis, called 'yersiniosis', which in many cases is due to consumption of contaminated foods. The pathogenesis of the bacterium is strongly associated with its enormous capacity to adapt to varying environmental conditions. This versatility enables the organism to multiply at temperatures near zero (e.g. in refrigerated foods), but also to switch over to a life at 37°C (e.g. in a warm-blooded host). This switch includes the expression of 'invasion factors' (to enable entrance of the host's tissues), and the production of serum resistance- and anti-phagocytosis factors (to restrain the host's immune response). In so doing, the bacterium can avoid the host's defense mechanisms, and may easily spread from the intestinal tract to other organs, which leads to a wide spectrum of serious post-infective extra-intestinal diseases and long-term sequela.**

Y. enterocolitica, **although first isolated in North America in the 1930s, emerged as an important enteric pathogen in much of the industrialized world in the 1970s and 1980s. There is a considerable geographic variation in the incidence of yersiniosis, ranging from < 0.0001% in low-incidence countries to ≥ 0.01% in high-incidence regions (<1 to ≥100 cases/year/ 1 million inhabitants).**

The gastrointestinal tract of swine is the main natural habitat of virulent strains. The improvement of slaughtering procedures (i.e. to prevent the contamination of carcasses), and the ameloriation of household hygiene (i.e. to repel the consumption of raw pork and the risk of cross-contaminations in the kitchen) have lead to a decline of yersiniosis, especially in the high-incidence countries, in the 1990s.

In addition to contracting yersinosis via the oral route, infection with the bacterium may also occur after transfusion with contaminated blood products, which leads in most cases to a life-threatening septicaemia. Transfusion-associated infections are not seldomly caused by blood products which had been obtained from asymptomatically infected donors, and which had been stored at 4°C for several weeks prior to the transfusion. Features like its psychrotrophic character, its ability to invade eukaryotic cells, its resistance to intracellular killing at low temperatures, and its capacity to benefit from iron enrichment due to aging erythrocytes, all contribute to proliferation of the bacterium under these conditions.

In conclusion, reduction of the contamination level of raw materials and subsequent chilling is not enough to restrain the cold-adapting pathogen *Y. enterocolitica*, neither in foods nor in blood products. Hence, additional methods are required to prevent the organism from unfolding its psychrotrophic character during storage at low temperature.

Yersiniosis: a foodborne disease

In september 1976, more than two hundred children at five schools in a restricted area of the USA fell ill with symptoms of abdominal pain, fever and diarrhoea, whereupon dozens of them were hospitalized and appendictomized upon suspicion of acute appendicitis. In the same year, two outbreaks of gastric infection with identical symptoms, involving ca. 150 schoolchildren, were reported in Canada. In 1981, over two hundred members of a summer camp population in the USA fell victim to the gastrointestinal symptoms described above, and the next year two outbreaks of the same kind affected more than a thousand people, spread over several states in the USA. Between 1972 and 1984, ten explosive outbreaks of a similar illness, each affecting several hundreds up to over a thousand children of rural primary schools and junior-highschools, occurred in Japan. Outbreaks of the same kind had previously been reported from nursery schools in Chezchoslovakia and, at a smaller scale, in families in Hungary and the USA, and in hospitals in Finland. All of these cases of human gastric infection had in common the fact that the causative agent identified was not one of the thus far commonly found enteropathogenic bacteria, but another species, called *Yersinia enterocolitica*. The disease was therefore called yersiniosis. An important resemblance in these outbreaks of yersiniosis was the presumed or proven implication of contaminated food as the source of infection.

1.1 HISTORY

1930s: First appearance

The organism presently known as *Yersinia enterocolitica* can be labelled as a 'recent' organism: its written history dates back only to 1934. In that year, a bacterium isolated from the facial ulcers of an American farm dweller was described that could not be identified as any

species known at that time, although its morphology and certain biochemical characteristics indicated "... *a similarity to the Pasteurella genus.*" [McIver & Pike 1934]. The isolate fell into oblivion for a couple of years, but attracted renewed interest in 1939, when at the New York State Department of Health three look-alike bacterial cultures were received that had been isolated from patients with life-threatening intestinal infections [Schleifstein & Coleman 1939]. The New York investigators thought these organisms, including a fifth identical culture from the NYSDH-collection (that had been isolated as early as 1923 from a chronic skin lesion in a carpet worker), particularly resembled *Pasteurella pseudotuberculosis*, a microbial species that had been known since the end of the last century to cause serious disease in animals. In the following years, annual reports of the New York State Health Department contained sporadic notes of the isolation of bacteria similar to those described in 1939 [Gilbert 1940]. Since these isolates mostly originated from children suffering from enteritis, the yet unclassified organism was temporarily called *Bacterium enterocoliticum* [Schleifstein & Coleman 1943].

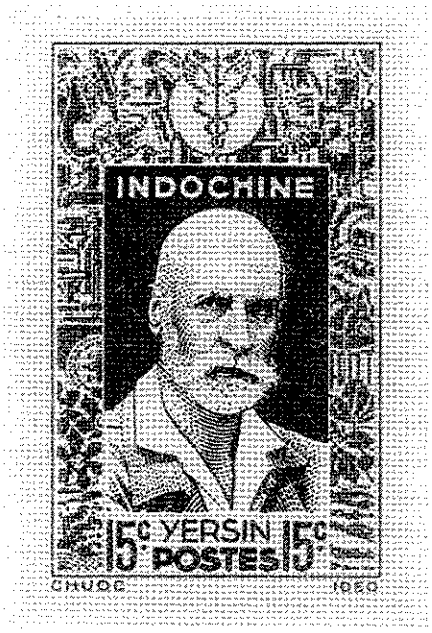
1940s - 1960s: Secluded existence

Little was heard about *B. enterocoliticum* for about twenty years, and from 1957 to 1968 complete silence surrounded the organism in the USA, when no cases of human infection due to it were reported. In the meantime, however, bacteria 'resembling *P. pseudotuberculosis*' were also recognized to be involved in human illness in Europe. The isolation of two such strains from patients who had died of septicemia was reported in Switzerland in 1949, whereupon the species was temporarily designated *P. pseudotuberculosis* ssp. *rodentium* [von Hässig *et al.* 1949].

1960s: Coming out again

In the early 1960's, the bacterium was recognized as an animal pathogen, when *P. pseudotuberculosis*-like bacteria were reported to be causative agents in enzootics among various wild and captive animals, including chinchillas in Europe and North- and Central America, hare in Europe, and pigs in North Africa [Akkermans & Terpstra 1963; Daniëls & Goudzwaard 1963; Mollaret 1964; reviewed by Hurvell in 1981]. At approximately the same time, the organism, then referred to as *Germe X* or *P. pseudotuberculosis X* or *type B*, was again shown to be involved in human infections, but now in Sweden, France, and Belgium [Carlsson *et al.* 1964; Mollaret & Destombes 1964; Winblad *et al.* 1966; Vandepitte *et al.* 1973]. In addition, various animal hosts, such as deer and pigs, frequently appeared to be healthy carriers of the bacterium [Dickinson & Mocquot 1961; Wetzler & Hubbert 1968].

From then onwards, this intriguing organism became the focal point of intensive investigations. In 1963/64, the similarity was established between the American and European strains of both animal and human origin bearing the aforementioned epithets [Knapp & Thal 1963], and it was then proposed to define this organism as a new species in the genus *Yersinia*, which in 1944 had been split off from *Pasteurella* [Frederiksen 1964]. The name *Yersinia* for this new genus had



previously been chosen to honour the French bacteriologist Dr. Alexandre Jean Emile Yersin who, in 1894, first isolated the infamous plague bacillus, which is now known as *Yersinia pestis* [Butler 1983; Solomon 1995]. The suffix *enterocolitica* refers to the organism's most frequent habitat in cases of human disease: the intestine and the colon.

Alexandre Yersin (1863-1943), shown on a stamp, issued in 1944 in Indochine. Even today, Yersin is a legendary figure in this region, the present Vietnam. He is still greatly hounoured, not only for his work in beating the plague but also for his many other contributions to improve the welfare of the Vietnamese people, including the introduction of the rubber tree (*Hevea brasiliensis*) and the quinine tree (*Cinchona leidgeriana*) in this part of the world.

1970s - 1990s: Expansion and establishment

Whereas by 1965 less then 30 cases had been reported world-wide [Weir 1985], over 600 reports appeared on the association of *Y. enterocolitica* with human disease during the second half of the 1960s [Morris & Feeley 1976]. Simultaneously, a rapid expansion of the geographical distribution of the bacterium was seen: it was rediscovered in the USA in 1968 [Sonnenwirth 1968], and at approximately the same time reported from the Netherlands [Wulf *et al.* 1969], Canada [Albert & Lafleur 1971], South Africa [Rabson & Koornhof 1973] and Japan [Zen-Yoji & Maruyama 1972]. Within a decade, the collection of the International Reference Centre at the Pasteur Institute in Paris comprised more then 6,600 strains covering 35 countries on six continents [Mollaret *et al.* 1979]. In the 1970s and 1980s, yersiniosis evolved into a serious threat to human health, when it was implicated in recurrent outbreaks of foodborne disease in Japan [Zen-Yoji 1981] and North America [Shayegani & Parsons 1987], and gained endemic character in north western Europe and some Asian regions [WHO 1981; Markov *et al.* 1989; Dmitrovsky *et al.* 1998]. To some extent, the steady increase in reported isolates of *Y. enterocolitica* obviously reflects the growing interest of microbiologists in this species. However, worldwide surveillance data show an explosion in the number of reported non-outbreak isolates and cases of yersiniosis in the last two decades. Obviously, the increased investigative activity can only marginally account for it, and this notice inclined several authors to refer to *Y. enterocolitica* as a worldwide emerging enteric human pathogen [Cover & Aber 1989; McCarthy & Fenwick 1990; Lee *et al.* 1991; Ostroff 1995; Tauxe 1997].

1.2 CLASSIFICATION

1.2.1 Characteristics

Yersinia enterocolitica is a Gram-negative, non-sporeforming, facultatively anaerobic rod of 1.3-3.5 x 0.5-1.0 μm in size. Based on morphological and overall biochemical characteristics, this bacterium belongs to the family *Enterobacteriaceae* [Bercovier & Mollaret 1984]. The optimum growth temperature of *Y. enterocolitica* is about 28°C, but the organism is able to multiply at 40°C, as well as at temperatures around zero [García de Fernando *et al.* 1995; Greer *et al.* 1995; Bergann *et al.* 1995; Miller *et al.* 1997]. The ability to grow at refrigerator temperatures is a feature shared with all the other members of the genus, including *Y. pseudotuberculosis* and the ill famed *Y. pestis* [Bercovier & Mollaret 1984; Gray 1995]. *Y. enterocolitica* is motile by means of several flagellae when grown in cultures at 30°C or less, but non-motile when grown at 35-37°C (Figure 1.1). Many other phenotypic characteristics, such as lipopolysaccharide (LPS) composition and virulence determinants like enterotoxin production and synthesis of secreted proteins (the so-called Yops), are temperature-dependent [Straley & Perry 1995].

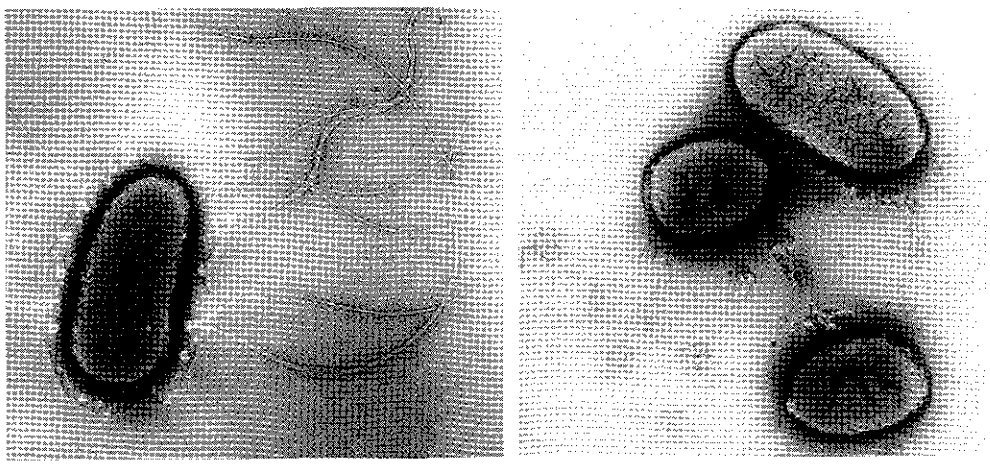


Figure 1.1 *Yersinia enterocolitica.* On the leftside: cells grown at 5°C; rightside: cells grown at 37°C

1.2.2 Related Species

Because the biochemical criteria initially proposed for the species *Y. enterocolitica* incorporated a rather heterogeneous group of bacteria of human and animal origin, several bio- and serotyping schemes have been developed to sub-group these isolates [Winblad 1967; Niléhn 1969; Wauters 1970; Knapp & Thal 1973]. Classification by these methods soon revealed certain

relationships between biotypes, ecological distribution and virulence [Alonso *et al.* 1976; Mollaret 1976; Mollaret *et al.* 1979]. Moreover, taxonomic studies applying DNA hybridization techniques elucidated differences in DNA-relatedness between typical *Y. enterocolitica* strains and those which were aberrant in phenotypic characteristics to such an extent that they had been referred to as '*Y. enterocolitica*-like' [Bercovier *et al.* 1980a; Brenner 1979; Brenner *et al.* 1976 1980a]. Hence, several groups of strains were reclassified as separate species and renamed *Y. intermedia* [Brenner *et al.* 1980b, *Y. kristensenii* [Bercovier *et al.* 1980], or *Y. frederiksenii* [Ursing *et al.* 1980]. The latter names were chosen in honour of the Danish microbiologists Kristensen and Frederiksen, who played important roles in the unraveling of the relationships of Yersinia-like organisms. In 1935, Dr. Martin Kristensen published a large study on so-called 'Paracolibacilli', originating from human faeces or urine [Kristensen *et al.* 1935], and in the 1960s one of these isolates was recognized as a Yersinia by Dr. Wilhelm Frederiksen. This strain was later chosen as the type strain for the newly defined species *Y. frederiksenii.*

When the relationships between the various strains became further unravelled, several other species were newly defined and - with reference to some other famous players in the continuing story of *Yersinia* - designated *Y. aldovae* [Bercovier *et al.* 1984], *Y. rohdei* [Aleksić *et al.* 1987], *Y. mollaretii* or *Y. bercovierii* [Wauters *et al.* 1988a]. Initially, these close relatives of *Y. enterocolitica* were very rarely associated with yersiniosis. However, their innocence is questionable, since several atypical cases of yersiniosis due to these strains have been described, more recently [Lewis & Chattopadhyay 1986; Cafferkey *et al.* 1993; Necrasova *et al.* 1998b].

1.2.3 Bio- and Serotypes

Despite the split off of the aforementioned related species, the bacteria currently classified as *Y. enterocolitica* by no means constitute a homogeneous group: yet several biotypes, based on their biochemical profiles, and a still increasing number of over 60 different serotypes, based on their somatic (O) and flagellar (H) antigens, can be distinguished [Wauters 1981; Wauters *et al.* 1991; Fenwick *et al.* 1996]. The situation is further complicated by the fact the H-antigens are species-specific, while the O-antigens are not [Aleksić 1995].

Although *Y. enterocolitica* was first recognized in relation to human illness, it soon appeared to be ubiquitous in nature: the bacterium has now been isolated from many vertebrate wild and domestic animals, from a variety of terrestrial and freshwater ecosystems, from drinking water and from raw and prepared food products [Mollaret *et al.* 1979]. As the number of isolates increased, a striking dichotomy was seen, with on the one side acknowledged pathogens and on the other side a range of so-called environmental strains. A fair correlation appeared to exist between biogroups, antigenic patterns and ecologic behaviour. Virulence was associated with only a dozen bio/-serotypes, whereas the vast majority of strains recovered from environmental sources were either non-typeable or serotypes which have never been implicated in human infections (Table 1.1, adapted from [Aleksić & Bockemühl 1990]).

Table 1.1 *Yersinia enterocolitica* bio- and serotypes which are regularly involved in human disease: ecological & geographical distribution and recognized transmission vehicles.

STRAIN TYPE		DISTRIBUTION		TRANSMISSION	
Bio type	**Sero-type**	**Ecological spread (main hosts)**	**Geographical spread (main areas)**	**Source / Vehicle in outbreaks or trans-fusion-acquired cases of yersiniosis**	**References**
1B	O:4	environment	USA/Canada, India	well-water? humans	*142* *417*
	O:8	pigs, dogs, rodents	worldwide (before 1980 only in the USA)	milk (-products) pre-cooked meat pork processing-water surface-water humans, pets	*77, 369, 376* *369* *220* *11, 59* *241* *191*
	O:13a,b	monkeys, environment	USA Europe	milk? well-water	*396* *261*
	O:18	environment	USA	well-water? milk?	*142* *396*
	O:20	dogs, rats	USA	pets blood	*435* *404*
	O:21	environment	USA/Canada	surface-water? milk? humans?	*276*
2	O:9	pigs, dogs, cats, rodents	Europe, Japan, Australia	pork humans, pets blood citrus-fruit	*342* *33, 417* *12, 404* *273*
	O:5,27	pigs	worldwide	pork, milk blood	*240* *404*
3	O:5,27	pigs	Europe, Asia	citrus-fruit	*273*
	O:1,2,3	chinchilla, pigs,	worldwide	humans? blood	*198,349* *404*
4	O:3	pigs, dogs, cats, rodents	worldwide (not in the USA before 1980)	pork, milk? processing-water well-water pets blood	*45, 262,263,401* *26* *124* *33* *12, 288,253,402*

In addition to the specific ecological spread of the distinct bio/serotypes, a certain geographical distribution was initially also manifest. One group of strains, i.e. the biotype 1B strains, comprising the serotypes O:4, O:8, O:13a/b, O:18, O:20 and O:21, were mainly isolated in the USA [Wilson *et al* 1976; Eden *et al.* 1977; Martin *et al.* 1982; Black *et al.* 1978; Shayegani *et al.* 1983; Tacket *et al.* 1984, 1985] and these were therefore referred to as 'American strains'.
On the other hand, the strains that were the most common causes of yersiniosis in Europe and Japan, i.e. serotypes O:3 and O:9, were virtually unknown from America. Only one pathogenic serotype, i.e. O:5,27, seemed to have a global spread from the very beginning. Since the early 1980s, however, the distinction between 'American' and 'non-American' strains no longer applies as a result of worldwide serogroup shifts, involving an increase in the proportion of formerly rare serotypes and a concomitant decline of others [WHO 1981; Bottone 1983; Neogi *et al.* 1985; Hoogkamp-Korstanje *et al.* 1986; Bottone *et al.* 1987; Lee *et al.* 1990]; and [Chiesa *et al.* 1991; Ichinohe *et al.* 1991; Prentice *et al.* 1991; Kontiainen *et al.* 1994; Stolk-Engelaar & Hoogkamp-Korstanje 1996][1].
Furthermore, evidence has been growing in the last decades that classification by bio- or serotyping may not always predict pathogenicity: whereas formerly only biotypes 1B, 2, 3, 4 and 5 were thought to be indicative for virulence, several sub-groups of biotype 1A have by now also been shown to be involved in human disease [Noble *et al.* 1987; Bissett *et al.* 1990; Greenwood & Hooper 1990; Burnens *et al.* 1996], especially among young children [Glenn Morris *et al.* 1991] and immuno-compromised persons [Sulakvelidze *et al.* 1998].

[1] In the first decades of *Y. enterocolitica* research, the strains which were pathogenic for humans could be divided in 'American' and 'non-American' strains, based on their restricted geographical distribution. Since the onset of the 1980s, however, the formerly uneven spread of certain serotypes has gradually flattened out. Serotype O:3, for example, which was rarely isolated in America until 1983, has since then increasingly been recovered from sporadic cases of yersiniosis in the USA. In 1990, this serotype was for the first time reported to be involved in an outbreak, and nowadays O:3 predominates in North America, whereas most of the serotypes implicated in early outbreaks are rarely seen.The reverse movement was seen with the 'American' serotype O:8. Such strains were initially unknown outside North America, but around 1985, isolations from human patients started to be reported from Asia and Europe. In fact, the first recorded case of yersiniosis in Bangladesh, which occurred in 1984, was due to infection with an O:8 strain. Although serotype O:8 is still very rare in Belgium and Scandinavia, it now forms approximately 4% of the recorded *Y. enterocolitica* isolations in the Netherlands and the United Kingdom. In addition to the arrival of the formerly absent serotype O:8, the initially uneven distribution of serotype O:9 over Europe gradually flattened out. In the British Isles, for example, serovar O:9 was unknown until 1980, but today, over 50% of the pathogenic strains isolated from human faeces belong to this serotype. Inversely, the proportion O:9 in Finland changed from 41% in 1974 to 1% in 1994, and this serotype is still extremely rare in Denmark.

1.3 CLINICAL MANIFESTATION

1.3.1 Gastrointestinal Syndromes

The clinical spectrum of *Y. enterocolitica* infections varies with age and underlying conditions [Bottone 1997]. The most common presentation of an orally acquired infection is a diarrhoeal disease, associated with low grade fever and abdominal pain, lasting for a few days to several weeks. This type of - usually mild - gastroenteritis is particularly found in infants and young children and is normally self-limiting. The symptoms can even be so faint and short-lived that yersiniosis it not diagnosed, despite faecal carriage [Ossel 1990]. Sometimes, however, the clinical course of the infection is much more serious and destructive. Syndromes like extensive ulceration of the intestine and subsequent peritonitis or an acute abdomen, due to invagination of the infected section of the intestine into a neighbouring part ('intussuspection'), are not uncommon in young children, and several fatalities have been reported [Gutman *et al.* 1973; Martin *et al.* 1982; Staatz *et al.* 1998]. In older children and adults, clinical syndromes known as 'terminal ileitis' and 'mesenteric lymphadenitis' are more common, which refer to strong inflammatory reactions in the distal small intestine and to swelling of regional lymph nodes [Bottone 1977]. Unlike infections with other common foodborne pathogens, yersiniosis frequently manifests itself with symptoms that mimic appendicitis, leading to sometimes unnecessary appendix operations [Shorter *et al.* 1998]. The upper part of the gastrointestinal tract may also be affected, leading to symptoms of pharyngitis [Gutman *et al.* 1973; Tacket *et al.* 1984]. In elderly people, or persons whose normal host-defense mechanisms have been compromised, the bacterium may persist in intestinal tissue, causing chronic inflammatory bowel diseases [Kallinowski *et al.* 1998].

1.3.2 Complications and Sequela

Due to subsequent spread of the bacterium via the blood stream, other body parts may become infected and a generalized, extraintestinal infection ('septicemia') may occur. Although this complication is especially found among people with an iron-overload [Piroth *et al.*, 1997; Adamkiewicz *et al.* 1998] or suffering underlying diseases [Jensen *et al.* 1995], septicemia may also occur in otherwise healthy persons [Hosaka *et al.* 1997]. Its manifestation includes not only relatively harmless skin inflammations [Gauthier & So 1997], but also abscess formation in liver and spleen [Schiemann 1989], as well as life-threatening infections of brain ('meningitis'), lung ('pneumonia'), heart ('endocarditis') and blood vessels ('aneurysm') [Challa & Marx 1980; Giamarellou *et al.* 1995; Donald *et al.* 1996; Mercié *et al.* 1996], and [Bin-Sagheer *et al.* 1997; Bottone 1997; La Scola *et al.* 1997; Tame *et al.* 1998].

As a result of the host's immune response, *Y. enterocolitica* may also induce secondary, post-infectious auto-immune diseases such as cutaneous granuloma on the extremities ('erythema nodosum') [Niemie *et al.* 1976; Schiemann 1989], and acute and chronic arthritis [Petrus *et al.* 1997;

Heyden *et al.* 1997], especially in adolescents and older adults possessing the tissue type HLA-B27 [Ahvonen & Rossi 1970; Larsen 1980; Falcão *et al.* 1995]. There is also substantial evidence that subclinical persistent infections with *Y. enterocolitica* may induce auto-immune thyroid diseases [Wenzel *et al.* 1996].

1.4 PATHOGENESIS

1.4.1 Entrance, Colonization and Spread

Historically, *Y. enterocolitica* is primarily a gastro-intestinal tract pathogen, although it has also emerged as a significant cause of blood transfusion-associated bacteraemia in the last two decades (see section 1.6.5). The sequence of events following ingestion of virulent *Y.enterocolitica* cells can be summarized by five steps: (i) invasion of intestinal epithelial cells, (ii) penetration of the lamina propria, (iii) multiplication in underlying tissues, (iv) drainage to mesenteric lymph nodes, and (v) entrance into the bloodstream eventually leading to systemic infection [Cornelis *et al.* 1987; Bottone 1997] (Figure 1.2).

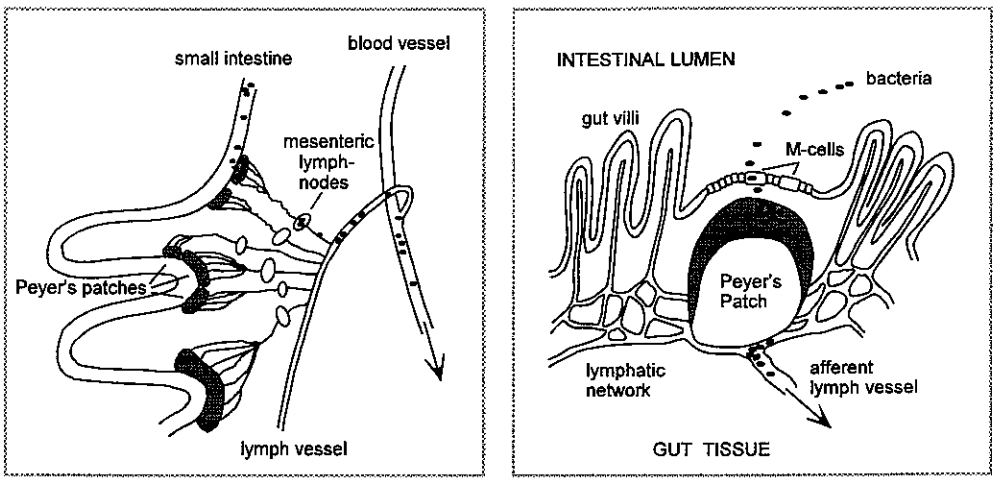


Figure 1.2 Entrance and spread of *Yersinia enterocolitica* in the human host

The first step, entry of the intestinal epithelium, is also seen in other entero-invasive bacteria, like *Shigella* and *Escherichia coli*, but while these enteropathogens usually invade the colonic epithelial layer, *Y. enterocolitica* preferentially localizes, like *Salmonella* species, to the distal

small intestine, the ileum. Secondly, *Y. enterocolitica* has, in contrast to other enteropathogens which usually remain and multiply locally in the epithelial cells, a strong propensity to penetrate the underlying lamina propria and to invade the gut associated lymphoid tissue, especially the organized lymphoid follicles known as Peyer's patches [Hanski *et al.* 1989]. Virulent strains of *Y. enterocolitica* can survive at this site, and multiply as extracellular microcolonies because they are able to resist phagocytosis by macrophages and polymorphonuclear leucocytes.
On the other hand, the organism can withstand intracellular killing by non-professional phagocytes. Hence, the bacterium can use leucocytes to translocate through endothelial monolayers [Rüssmann *et al.* 1996], thus allowing them to drain from the Peyer's patches into lymphatic vessels and to colonize the regional lymph nodes, the liver, and the spleen. Eventually, entrance into the bloodstream may lead to further spread of the infection, inducing various systemic diseases and immunologically mediated sequelae.
Based on their role in the various steps of pathogenesis, several types of virulence factors can be distinguished in *Y. enterocolitica.*

1.4.2 Virulence Factors

Invasion factors (Figure 1.3)
The fist steps of infection - adherence to and invasion of the epithelial layers of the host gut - require at least two chromosomal factors, called *ail* (for Adhesion Invasion Locus) and *inv* (for invasion) [Miller & Falkow 1988].
The *inv* gene is present in virulent as well as in non-virulent strains, whereas the *ail* gene is only found in pathogenic serotypes of *Y. enterocolitica* and in *Y. pestis* and *Y. pseudotuberculosis*, [Miller *et al.* 1989]. The *inv* product of *Y. enterocolitica*, invasin, is a ca. 90 kDa outer membrane protein that mediates cellular entry by binding to integrin receptors on the surface of certain epithelial cells, the so-called M-cells [Pierson 1994]. These cells, which cover the Peyer's patches, are specialized in delivering internalized particles to the underlying macrophages, and the bacterium thus exploits this host cell function to pass through the cellular barrier of the intestinal epithelium and invade the underlying tissue [Finlay & Cossart 1997]. The mode of action of the 17 kDa *ail* product, Ail, which is also an outer membrane protein, has not been elucidated so far.
In addition to the chromosomal factors, at least one extrachromosomally encoded factor directly contributes to invasion. This factor, formerly known as POMP1, P1 or YopA (for Yersinia Outer Protein A) [Cornelis *et al* 1987 1989] but now generally referred to as YadA (for Yersinia adhesin), is the product of the *yadA* (or *yopA*) gene, which is one of the genes present on pYV, the Yersinia virulence plasmid (see below). YadA consists of subunits of about 50 kDa and forms a fibrillar structure on the surface of the bacterium, which, among other functions, mediates clumping and adherence to intestinal mucin [Straley & Perry 1995].

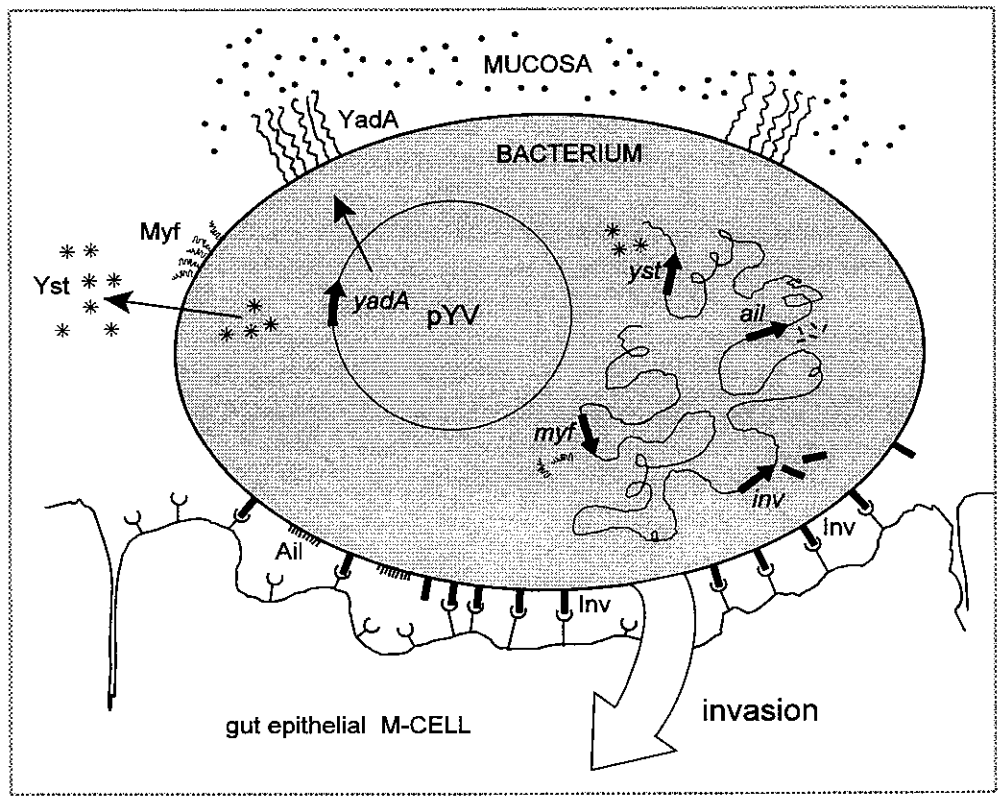


Figure 1.3 Yersinia enterocolitica in the intestine:
genes and gene-products which are involved in the invasion of the host epithelial tissues

Anti-phagocytosis factors

After invasion of the intestinal mucosa, the bacterium has to defend itself to the non-specific immune response of the host, especially to phagocytosis by polymorphonuclear leucocytes The anti-phagocytosis strategy relies mainly on a dozen secreted proteins, called Yops (for Yersinia outer protein's), their individual cytosolic chaperones, called Syc proteins (for Specific Yop chaperone), a dedicated secretion apparatus which is made up of a twenty Ysc (for Yop secretion) proteins, and several regulatory proteins [Cornelis 1994; Boland *et al.* 1996].

Both the *yop*, *syc* and *ysc* genes, as well as the *vir* genes that regulate their expression, are found on a high molecular weight (70-75 kb) plasmid called pYV (for Yersinia Virulence) [Cornelis et al. 1987 1989; Bliska 1994; Iriarte & Cornelis 1998]. This plasmid is highly conserved among all virulent *Yersinia* species and serotypes, and pathogenicity is lost upon loss of pYV [Gemski

et al. 1980; Schiemann & Devenish 1982; Heesemann *et al.* 1983; Portnoy & Martinez 1985]. Cells which harbour pYV require Ca^{++} for growth at 37°C. In the absence of calcium ions, virulent Yersiniae restrict their growth at 37°C and synthesize, instead, large amounts of Yops. This phenomenon reflects a phase transition that allows the bacterium (i) to adapt to its environment - the infected host - and (ii) to proceed with the successive steps in infection.

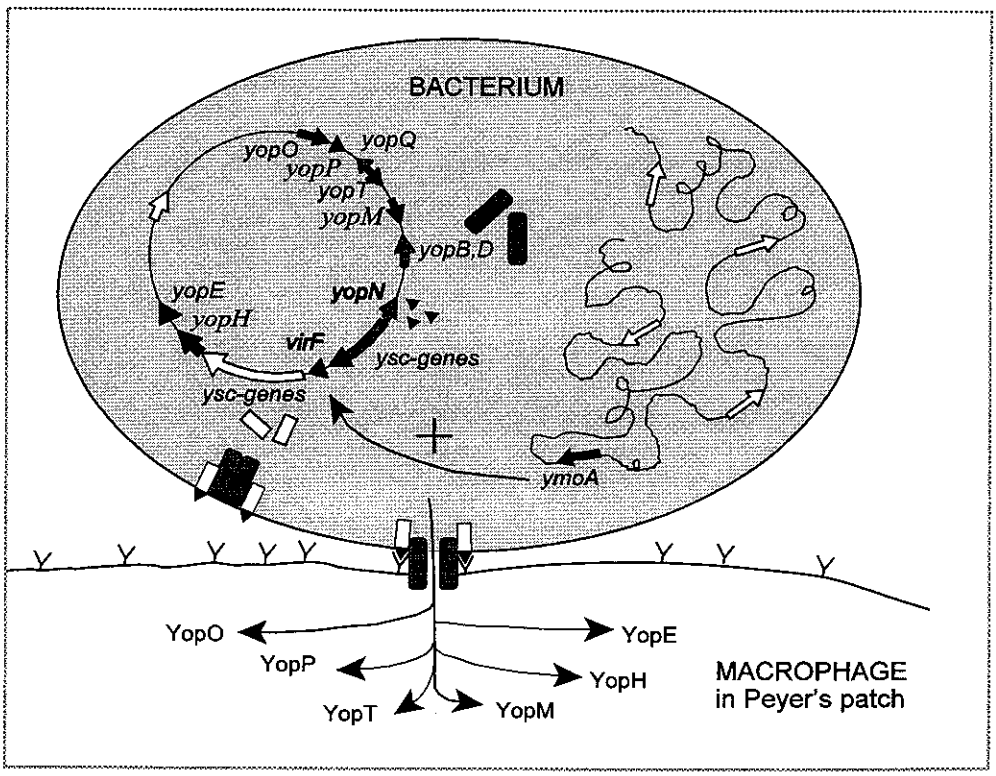


Figure 1.4 *Yersinia enterocolitica* in the Peyer's patch: genes and gene-products involved in the process of anti-phagocytosis.

The mode of action of several Yops has now largely been elucidated and a model has been proposed to explain the interaction between *Y. enterocolitica* and the target cell [Cornelis & Wolf-Watz 1997] (Figure 1.4). Upon contact of bacteria with the host cell surface, the membrane associated proteins YopB and D act, in co-operation with the membrane-bound Ysc secretion system, as a translocation apparatus to inject YopE, H, M, O, P and T into the phagocytic cells [Rosqvist *et al.* 1994; Persson *et al.* 1995; Boland *et al.* 1996; Mills *et al.* 1997; Iriarte & Cornelis 1998]. YopN

exerts, probably as the sensor of Ca^{++} and/or by binding to a specific receptor on the eukaryotic cell, a control function on assembly of the delivery apparatus [Cornelis 1994; Cornelis & Wolf-Watz 1997]. After internalization, YopH, M and O interfere with signal transduction pathways, thus inhibiting the functioning of the host cell, while YopE and YopT damage the infected cell by paralysing its actin cytoskeleton, and YopP induces programmed cell death (apoptosis) [Mills *et al.* 1997].

Serum resistance factors

In addition to favouring invasion and/or extracellular survival of the bacterium, some of the above proteins also interfere with other, blood-related, non-specific host defence mechanisms. Yop M, for example, acts as a serum resistance factor by binding to thrombin, thus inhibiting thrombin-induced platelet aggregation [Bliska 1994]. Similarly, YadA prevents the formation of the membrane attack complex of complement by binding of the complement factor H [Cornelis 1994]. There is some evidence that Ail also contributes to serum resistance, but its mode of action is still unknown [Bliska & Falkow 1992].

Serum resistance is also thought to play a role in transfusion acquired *Yersinia* infections. While serogroup O:8 strains present in leucocyte-filtered human blood are rapidly killed by the serum bactericidal activity at 25°C or during storage at 4°C, serotype O:3, O:5 and O:9 strains survive 25°C holding temperatures and ultimately multiply during storage at 4°C [Bottone 1997].

Auto-immune response inducing factors

The mechanisms promoting auto-immunity and chronicity in arthropathies are far less understood than those of the acute illness. Most patients manifesting post-*Yersinial* reactive arthritis are HLA-B27 positive, but the reasons underscoring this predisposition are still obscure [Märker-Hermann & Höhler 1998]. Nevertheless, several positively charged Yersinia proteins, such as O-polysaccharide antigens and the small ß-subunit of urease, are suspected of playing a role in Yersinia-triggered reactive arthritis [Bottone 1997]. Such cationic proteins might easily bind to the negatively charged structures of the joint cartilage or synovial lining. Indeed, O-polysaccharide antigens have been detected in synovial fluid cells from arthritic patients [Granfors *et al.* 1989; Viitanen et al. 1991], and the urease-subunit was shown to be an immunodominant target antigen for synovial T-lymphocytes [Mertz *et al.* 1994].

Enterotoxins

Y. enterocolitica and related species produce a heat-stable, chromosomally encoded enterotoxin (Yst, or YEST) when the bacteria are cultured in typical bacteriological media at 20-30°C [Pai *et al.* 1978]. It has long been doubted whether the enterotoxin is an element of virulence because absence of its in vitro production at temperatures above 30°C suggested that this toxin is not produced in the host intestinal lumen. More recently, however, it was shown that high osmolarity and weakly alkaline pH, conditions that are present in the normal ileum

lumen, allow significant transcription of *ystA*, the major Yst gene, at 37°C [Mikulskis *et al.* 1994], and this might also occur with two newly detected enterotoxin genes, *ystB* and *ystC* [Huang *et al.* 1997]. Furthermore, after *ystA* was cloned and sequenced [Delor *et al.* 1990], the implication of Yst in the onset of diarrhoea in infected rabbits could be demonstrated [Delor & Cornelis 1992]. In addition, a surface antigen called Myf (for Mucoid yersinia factor; see Figure 1.3) might be a colonization factor acting in conjunction with Yst to cause diarrhoea, because it closely resembles enterotoxin-associated fimbriae described in other bacteria, and presence of the *myf* genes (like that of *yst*) is restricted to pathogenic serotypes [Iriarte *et al.* 1993]. Furthermore, both Yst and Myf are produced only when bacteria enter the stationary growth phase [Iriarte *et al.* 1995].

In addition to putative enterotoxin production in the infected host, intoxication by means of preformed enterotoxin can not be excluded: YEST is also produced at 4°C, in foods and drinks [Boyce *et al.* 1979; Kapperud & Langeland 1981; Olsvik & Kapperud,1982] as well as in blood products [Bradley *et al.* 1997] and its activity is not destroyed by heating or chilling during foodprocessing [Francis *et al.* 1980], nor by the influence of gastric acidity [Boyce *et al.* 1979].

Iron-scavenging factors

Since iron is an essential nutrient for almost all microorganisms, *Y. enterocolitica*, like other pathogenic bacteria, must obtain iron from the host to establish an infection. In the mammalian tissues and intestinal environment however, the abundantly present iron is not readily available for bacteria since it is tightly bound to eukaryotic proteins such as haemoglobin, ferritin, transferrin and lactoferrin. Therefore, the ability to capture iron in vivo is one of the critical factors that differentiate high- and low- pathogenicity *Y. enterocolitica* strains.

In response to iron limitation, the highly pathogenic 'American' biotype 1B strains O:4, O:8, O:21 and O:40 synthesize both an iron-chelating molecule, designated Yersiniabactin, and a specific outer membrane receptor for Yersiniabactin, i.e. FyuA (for ferric yersiniabactin uptake) [Heesemann 1990; Chambers & Sokol 1994]. The production of Yersiniabactin, is regulated by HMWP2, a high molecular weight protein encoded by *irp*2, which is also synthesized only by high-pathogenicity strains, under iron-stress conditions [Carniel et *al.* 1992; Guilvout *et al.* 1993]. All other *Y. enterocolitica* strains, including the serotypes O:3, O:5,27 and O:9, do not produce Yersiniabactin, nor any other 'siderophore'. Nevertheless, these strains can, to a certain level, also fill their iron need, since they do express in their membrane various receptors for siderophores produced by the host or by other intestinal bacteria (like YfuA for polyphosphate-bound ferric iron, FoxA for ferrioxamines, FcuA for ferrichrome, and Hem R for haemin) [Straley & Perry 1995; Heesemann *et al.* 1997]. The fact that several of these siderophores (i.e. ferrioxamine B) are used to treat patients with an iron overload might explain why systemic *Y. enterocolitica* infections, which are commonly encountered in such patients, are mostly due to O:3, O:5 or O:9 strains [Robins-Browne *et al.* 1987; Carniel 1995; Piroth *et al.* 1997].

1.4.3 Temperature-dependent Gene Regulation

During its pathogenic life cycle, *Y. enterocolitica* transits from environmental niches into the tissues of the host. To avoid inappropriate virulence gene expression, the bacterium has developed a sophisticated regulation of its pathogenicity determinants in response to temperature stimuli. *Inv* and *yst* are expressed well at 30 °C and at lower temperatures, but their expression and/or activity drops at the mammalian host temperature of 37 °C, under the influence of a common factor, the chromosomal *ymoA* (for 'Yersinia modulator') gene [Pierson 1994; Cornelis 1994; Straley & Perry 1995]. In contrast to this downregulation, the expression of many other virulence genes, including *ail*, *yadA*, and the *yop*, *vir* and *ysc* genes, is largely enhanced at 37 °C, under direct or indirect control of *ymoA*. Actually, *VirF* functions as transcriptional activator of the *yop* genes, after its own induction by the histon-like protein YmoA.

1.5 EPIDEMIOLOGY

Y. enterocolitica has emerged as an important enteric pathogen, associated with a wide spectrum of clinical and immunologic manifestations, in much of the industrialized world. Nevertheless, the true incidence and prevalence of *Y. enterocolitica* infections are not known. In general, the incidence rate, that is the total occurrence of new cases among a certain population during a certain period of time, is estimated from the reported number of cases. In addition, estimates of prevalence, that is the proportion of the population having the disease at a certain time point, are derived from the faecal carriage of the bacterium among patients with gastro-enteritis, or from the prevalence of specific antibodies in the population.

1.5.1 Incidence

Since yersiniosis is a notifiable disease in only a few countries, our knowledge about its global incidence is far from complete. Nevertheless, the scattered data (Table 1.2) show that there are a few high-incidence regions in the temperate zones and many countries with a low to medium incidence.

Europe

In several West-European countries, especially in Scandinavia and Belgium, yersiniosis shows endemic character. At the end of the 1970s, it was the most frequent enteric infection in **Denmark**, and today, at the end of the 1990s, *Y. enterocolitica* is the third most common enteropathogen, after *Salmonella* and *Campylobacter* [Nielsen & Wegener 1997], a situation that is also seen in **Belgium** and **Sweden** [Verhaegen *et al.* 1991; Borch *et al.* 1996]. In medium-incidence countries, like **Norway, the Netherlands** and **the United Kingdom**, the bacterium now ranks

as number four after *Salmonella*, *Campylobacter* and *Shigella* [Nesbakken & Skjerve 1996; Esveld *et al.* 1996; Adams & Moss 1995]. In most of these countries, the incidence of yersiniosis peaked in the mid-eighties, after which it declined progressively (Figure 1.5), probably due to improved food hygiene at the industrial as well as at the household level [Nesbakken & Skjerve1996; Verhaegen *et al.* 1998].

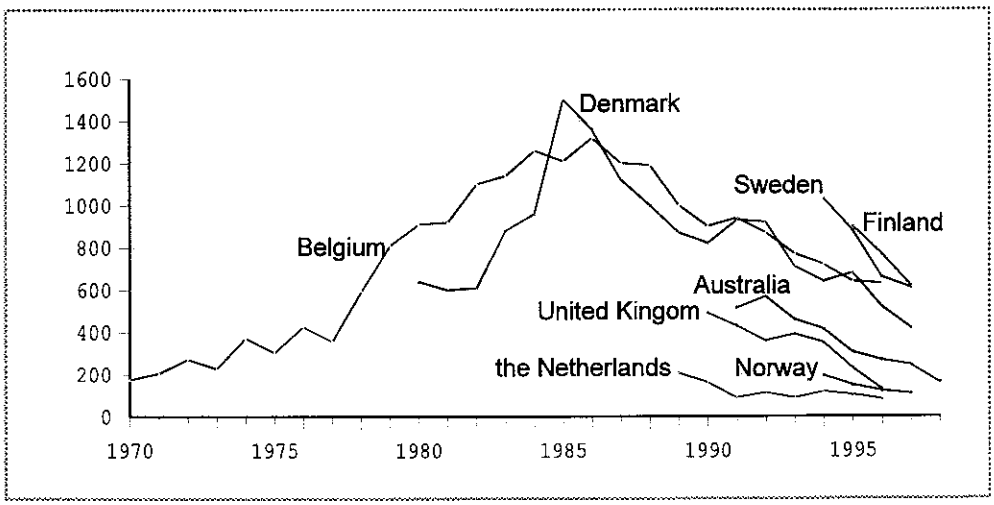


Figure 1.5 Trends in the incidence of yersiniosis:
amounts of annually reported cases in various countries

In the UK, the epidemiology seems to be somewhat different from that on the continent: whereas in the Scandinavian countries and Benelux nearly all yersiniosis concern sporadic cases, small outbreaks are much more regularly reported to occur in Great Britain [Cheasty *et al.* 1998]. Published data concerning yersiniosis in other European countries are scarce and/or out-of-date. Nevertheless, the disease seems to be less common in Central and Southern Europe, although strong geographic differences are seen, even between neighbouring countries (confer **France** versus **Spain**, or **Germany** versus **Switzerland**). Medium to high incidences have been reported in the 1970s and 1980s from several East-European countries, such as **Hungary** and the former **Czechoslovakia**, but recent data are not available. Absence of such data does not necessarily mean that yersiniosis has become less important in these regions, as is evident from the fact that several large epidemics have been reported to occur in **Romania** in the past decades, although incidence data have not been published [Constantiniu *et al.* 1998].

North America

Y. enterocolitica infections are also frequently reported from North America.

In the 1970s and 1980s, the epidemiology of yersiniosis in the USA and Canada was characterized by recurrent foodborne outbreaks, each affecting hundreds to thousands of people, mostly children [Schiemann 1989]. In the 1990s, outbreaks are still reported from these countries, but the number of patients is generally restricted to a few dozens [DeBuono 1995; Shorter *et al.* 1998]. This does not automatically imply that the disease is over the hill in this part of the world, for sporadic cases of foodborne disease - although less likely to be reported - are far more common than are cases that are part of recognized outbreaks [Bean & Griffin 1990]. Furthermore, under-reporting is very likely, since surveillance of foodborne disease is largely a voluntary system. Consequently, very low annual incidence rates (i.e. 0.2-0.4 cases per 1 million inhabitants) have been calculated from the amount of nationwide reported cases in the **USA.**, [Bean & Griffin 1990], whereas much higher incidence rates are found in regions with a reporting obligation, such as California and New York State, i.e. 2-5 cases per 1 million inhabitants [Bissett *et al.* 1990; Shayegani *et al.* 1995]. In the period 1973-1987, the bacterium ranked number ten after *Salmonella*, *Shigella*, *Staphylococcus aureus*, *Clostridium perfringens*, *Campylobacter jejuni*, *Streptococcus*, *E. coli*, *Bacillus cereus* and *Vibrio cholera* as causative agent in sporadic cases of foodborne disease of known etiology in the USA [Bean & Griffin 1990]. However, despite its relatively low ranking, *Y. enterocolitica* is considered to be a serious and still increasing problem in North America. Whereas the American Centers for Disease Control (CDC) estimated in 1985 that 5000 cases of yersiniosis would occur annually in the USA [Bennett *et al.* 1987], this estimate was increased to 17,000 in 1991 [CDC 1998a]. Since 1996, *Y. enterocolitica* is one out of seven bacterial agents for which CDC developed an active surveillance network (FoodNet), in order to better understand and respond to foodborne illnesses in the USA [CDC 1998b]. This surveillance, which now covers approximately 30 million persons (more than 10% of the US-population), has shown that the incidence of yersiniosis changed little from 1996 to 1998 [CDC 1998b].

In **Canada**, *Y. enterocolitica* has rivaled Salmonella and Campylobacter as a cause of acute gastroenteritis since the end of the 1980s, being reported from both isolated cases and small-scale outbreaks [Cover & Aber 1989; Harnett *et al.* 1998].

South America

No pronounced history of yersiniosis is known from South America, but data which were produced from studies among childhood populations in **Chile** suggest that yersiniosis is also much more common on this continent than might be assumed from the virtual absence of reported cases [Glenn Morris *et al.* 1991].

Table 1.2 The occurrence of human yersiniosis in various countries: average annual incidences (cases per 1 million inhabitants)

COUNTRY	untill 1970	1970-1979	1980-1989	1990-1994	1995-1999	REFERENCES
Belgium	4	38	123	90	70	*413, 420, 421*
Chech-Slov.	47	.	40	.	.	*36, 348*
Crete	.	.	.	65	.	*361*
Denmark	.	40,000*	200	153	96	*47,125, 257, 305*
France	<1	< 1	.	.	>10	*44*
Finland	.	39	.	166	117	*1, 32, 169*
Germany	.	.	.	2	.	*41*
Hungary	3	36	.	.	.	*394, 434*
Italy	.	< 1	2	.	.	*122*
Lithuania	.	.	.	.	10*	*256*
Netherlands	.	53*	34*- 80*	13	9	*148, 149, 206, 207, 210*
Norway	.	.	57	46	25	*230, 317, 318, 331*
Spain	.	.	25	.	.	*182*
Sweden	13	.	80*	113	80	*86, 377, 437*
Switzerland	.	.	.	14	8	*60, 61, 104*
UK	.	< 1	6	10	3	*28, 121, 344, 429*
Canada	.	3	.	17	17	*198, 406*
USA	.	< 1	1*- 20*	5	10 - 65*	*64, 66, 75, 115, 116, 248, 368*
Chile	.	7	13	.	.	*181*
South Africa	.	7*	16*- 30*	.	.	*212, 228, 352*
Israel	.	35*	.	.	.	*375*
Georgia	.	.	6	.	.	*391*
Australia	.	.	.	28	14	*118*
Nw Zealand	.	.	157*	93 - 150	153	*155, 156*
Yakutia	.	73	73	73	.	*143*
Kazakhstan	.	.	.	.	27*	*283*
Japan	.	5*	.	.	.	*233*
Bangladesh	.	.	< 1	.	.	*105*

In general, the incidence-data are calculated from the amount of culture-proven cases, as reported to National Reference Centers, or published in occasional reports. Data marked with an asterisk (*) are either estimated by a National Health Office or based on studies in restricted areas. Dots in the list mean that no data were available.

Africa and Middle-East

Very few documented cases are known from developing countries in Africa. This could be due to lack of compulsory reporting, or yersiniosis is probably not a significant cause of enteric infection in these countries. Reports from **South-Africa** [Househam *et al.* 1988] and **Israel**, [Shmilovitz & Kretzer 1978] on the other hand, indicate that *Y. enterocolitica* infections are not uncommon in the more industrialized regions.

Oceania

Y. enterocolitica is nowadays one of the more commonly isolated bacterial enteric pathogens in **New Zealand**. Until 1988, yersiniosis was rare in this country but in the 1990s, the number of isolates was reported to be rising, and in some areas *Yersinia* now ranks second or third after *Campylobacter* and/or *Salmonella* [Fenwick & McCarthy 1995]. In **Australia**, on the other hand, a gradual decrease in the incidence of yersiniosis has been observed since it became a notifiable disease in 1991 [CDN Australia/New Zealand 1998].

Asia

Although incidence data are largely absent from Asian countries, yersiniosis is far from uncommon in certain areas. Large, most likely foodborne, outbreaks were reported to occur in Japan between 1972 and 1984 [Zen-Yoji 1981]. More recently, a lingering epidemic, with over 1000 cases (1% of the population!) each year, was reported to infest a certain region of **East-Kazakhstan** from the early 1990s onwards [Dmitrovsky *et al.* 1998]. In addition, small outbreaks have been reported from several other Asian countries such as **Mongolia** [Markov *et al.* 1989], **China** [Anonymus 1987] and **India** [Abraham *et al.* 1997].

1.5.2 Faecal Carriage in Humans

A second source of information that is used to estimate the actual amount of foodborne disease caused by a certain species is the number of isolates from clinical specimens obtained from patients under medical care for gastro-intestinal disorders. Data concerning faecal carriage of pathogenic strains of *Y. enterocolitica* are shown in Table 1.3.

Europe

The most complete picture is from **Belgium**, based on the number of isolations from hospital stools between 1970 and 1992. Until 1980, a steady increase is seen in the percentage of coprocultures positive for pathogenic *Y. enterocolitica*, which is thought to be due to improved isolation techniques and higher awareness of the pathogen [Verhaegen *et al.* 1998]. After peaking at 3.5% around 1980, a progressive decline in faecal carriage rate is seen from 1984 onwards [Van Noyen *et al.* 1987a, 1987b, 1995]. A similar pattern of rise and fall in the percentage of *Y. enterocolitica* in coprocultures is seen in **the Netherlands** and in **Italy**. But genereal trends are difficult to deduce since (i) published data are scarce and (ii) investigators have not always

distinguished between pathogenic and non-pathogenic strains. Evaluation of the data is further complicated by the bias that might be introduced by the selection criteria used to define patients and for taking samples in culture [Hoogkamp-Korstanje *et al.* 1986; Esveld *et al.* 1996]. In a Dutch study, for example, a higher faecal carriage rate (2.9%) was observed when every diarrhoeal stool sample sent to a Public Health Laboratory was cultured for *Y. enterocolitica* instead of only those which were suspected upon diagnosis by a general physician versus (1.6%) [Hoogkamp-Korstanje *et al.* 1986]. Furthermore, surveys in both **Italy** and **Sweden** showed that rather different levels of faecal carriage were observed between childhood out-patients and patients hospitalized for gastroenteritis, i.e. 0.6% versus 3.7% and 1.5% versus 5.6% [Figura & Rossolini 1985; Uhnoo *et al.* 1986]. Such bias factors might also play a role in the aberrantly high faecal carriage rate that has been reported from certain areas in the **United Kingdom.**

America

In the American countries with a pronounced history of yersiniosis, i.e. the **USA** and **Canada**, the faecal carriage rate has also declined since the mid-1980s. Surprisingly, pathogenic strains are also regularly isolated from diarrhoeal stools in regions where *Y. enterocolitica* is less prominent as a cause of enteric infection, such as **Chile** [Glenn Morris *et al.* 1991].

Africa and Middle-East

Very low isolation rates are found in **Israel** [Shmilovitz & Kretzer 1974], in Moslem countries such as **Iran** and **Nigeria** [Haghighi 1979; Anyanwu 1995], and in tropical African countries such as **Senegal** and **Kenya** [Franzin *et al.* 1987; Turkson *et al.* 1988]. In **South-Africa**, on the other hand, the bacterium is less rare. However, large differences can be found between different age groups. In one study, for example, the overall isolation rate of the bacterium in cases of acute gastroenteritis was found to be 0.1% [Baxter *et al.* 1994], but a ten-fold higher faecal carriage was observed among a sub-population of diarrhoeic children [Jennings *et al.* 1987; Household *et al.* 1988].

Asia

High isolation rates have been reported from **Japan** in the 1970s and 1980s, but today the faecal carriage seems to be much less. In **India**, where *Y. enterocolitica* is surpassed by many other, more virulent pathogens, the bacterium has been isolated from up to 3% of loose stools [Ram *et al.* 1987]. Much lower isolation rates are reported from other Asian countries, like **China** [Zheng & Xie 1996] and Bangladesh [Samadi *et al.* 1982; Butler *et al.* 1987].

1.5.3 Prevalence of Antibodies in the Population

A third criterion used to estimate the frequency of a certain bacterial infection (including subclinical cases) in a population, is the prevalence of specific antibodies. This is, however, a subject full of pitfalls, since the diagnostic value of a single titre not only depends on multiple host variables, such as age, underlying disorders, or the administration of immunosuppressive

Table 1.3 Human carriage of pathogenic *Yersinia enterocolitica*:: % of culture proven infections in diarrhoeal stools

COUNTRY	1970-1980	1980-1984	1985-1989	1990-1999	REFERENCES
Belgium	1.0	1.2 - 3.5	2.1	0.2	*414,415,416,421*
Chech/Slov.	0.1	.	.	.	*337*
Crete	.	.	.	0.7	*361*
France	(< 0.1)*	.	.	.	*301*
Finland	2.0	.	.	1.1	*32, 247*
Germany	0.1 - 1.5	.	.	.	*62, 80*
Italy	.	(0.3) - 1.4 (3.1)	0.6 - (3.2)	0.4	*100, 109, 158, 159, 295*
Netherlands	1.0	1.6 - 2.9	2.1 - (2.4)	0.2 - 0.7	*148, 149, 210, 389, 439*
Romania	10	(1.8)	(1.8)	(1.8)	*126, 357*
Spain	.	0.8	1.0	.	*182, 190*
Sweden	.	1.5 - (3.1) 5.6	.	.	*393, 410*
Switzerland	.	.	.	0.4	*104*
UK	.	.	0.3 - (3.5)	7.2	*185, 186, 265*
Canada	(0.2) - 2.6	1.8 - (5.3)	.	.	*274, 301, 324,*
USA	2.1	3.3	0.8 - (1.6)	.	*248,263,370,430*
Chile	.	.	0.9 - (1.4)	.	*181*
South Africa	0.1	(0.9) - 1.0	.	(0.1)	*63, 212, 228,352*
Nigeria	.	.	.	0.0	*51*
Senegal	.	.	0.0	.	*165*
Kenya	.	.	0.0	.	*409*
Malaysia	.	0.0	.	.	*227*
Zaïre	0.4	.	.	.	*271*
Iran	< 0.1	.	.	(2.0)	*192, 382*
Israel	< 0.1	.	.	.	*375*
Georgia	.	.	(1.0)	.	*391*
Australia	< 0.1	0.9	0.9	.	*275*
Nw Zealand	.	.	.	0.4 - (0.7)	*156, 402*
Japan	1.6 - 4.0	0.8 - 1.8	.	0.4	*171, 233, 234, 277, 443, 444*
India	.	(0.2) 0.6 - 3.0	.	.	*349, 379, 417*
Bangladesh	.	< 0.1	0.1	.	*105, 311, 359*
China	.	.	.	0.4	*445*

* Figures in brackets refer to studies in which the isolates were not bio-/ or serotyped; these may also include strains that are commonly not associated with disease.

agents [Bottone & Sheehan 1983], but also on the type of test used and the antibody background in the healthy population, for antibodies - especially those of the IgA type - may persist for months or even years after infection [Larsen *et al.* 1985; Paerregaard *et al.* 1991].

Diagnostic value of various agglutinin titres

The reference standard for measurement of *Yersinia* antibodies is a tube-agglutination test, but enzyme-linked immunosorbent assays (ELISA) are also used [Benoit *et al.* 1996]. In general, agglutinins titres of dilutions ≥1:128 are supposed to be diagnostic in a previously normal, healthy subject, whereas medium (1:80 up to 1:128) and low (≤1:40) level titres are presumably indicative of, respectively, recent and less recent back contacts with the antigen.

Background in the population

A few studies have been done to investigate the antibody background in a normal population, with varying results. In Italy, *Y. enterocolitica* antibody medium titres were found in 0.2% (anti O:3) or 1.3% (anti O:9) of general blood donors [Tamburrino *et al.* 1993; Franzin & Curti 1993]. Similar figures were obtained in the USA, i.e. 1.4% with low titres against O:3, O:5,27 and/or O:8 [Bottone & Sheehan 1983], and in France, i.e. 0.8% with low titres against O:3, and 2% with high titres against O:9 [Mollaret 1983]. However, the prevalence of antibodies may vary between sub-populations, as appears from two Irish studies in which it was shown that 2% of a control population of non-yersiniosis hospital patients had high titres, whereas 0% of general blood-donors had titres above 1:40 [Attwood *et al.* 1987; Cafferkey & Buckley 1987]. From a Finnish study, by contrast, it was concluded that a high frequency of *Yersinia* antibodies can be found in healthy populations: in the sera of 19% of Finnish and 33% of German blood donors, enhanced levels of O:9/O:3 specific antibodies were found [Mäki-Ikola *et al.* 1997].

High titres in sub-populations

Elevated serum antibody concentrations have been found among people involved in swine breeding or pork production. In Finland, for example, slaughterhouse workers and pig farmers were observed to have elevated antibody levels to *Y. enterocolitica* O:3 twice as frequently as grain- or berry farmers or randomly selected blood donors [Merilahti-Palo *et al.* 1991; Seuri & Granfors 1992]. Significant differences for antibody titres against pathogenic serotypes were also observed between abattoir personnel and general blood donors in Italy, i.e. titres of ≥1:40 in 19% versus 8% of persons, respectively [Franzin & Curti 1993; Franzin *et al.* 1998]. Likewise, similar differences were observed between office personnel and people practically involved in swine slaughtering in Norway [Nesbakken *et al.* 1991].

Practical use

Despite the above drawbacks, serology was shown to be a useful diagnostic tool in acute intestinal disorders: while, in a Swedish case-control study, a significant increase in *Ye* O:3-specific IgM antibodies was found in 85% of children with gastro-enteritis, only 2.9% of them (and none of the controls) shed *Y. enterocolitica* in their stools [Uhnoo *et al.* 1986]. Serology also

appeared to be a useful tool for diagnosis of sub-acute post-infectious complications, such as *Yersinia*-triggered reactive arthritis. In Italy, 33% of children suffering reactive arthritis were found to have medium or high (≥ 1:80 to 1:1280) anti-O:3/0:9 IgM titres [Taccetti *et al.* 1994]. Likewise, antibody titres 1:80 to 1:320 against *Ye* O:3, O:8, O:5 or O:6 were found in 19% of adolescent or adult patients with inflammatory joint diseases in Italy [Tamburrino *et al.* 1993].

1.6 TRANSMISSION ROUTES

Human yersiniosis is in many cases due to consumption of contaminated foods, quite often after previous storage of the vehicle at refrigeration temperatures. In addition, contaminated drinking water, contacts with human patients or infected animals, as well as blood-transfusions, have been recognized as modes to acquire a *Y. enterocolitica* infection (Figure 1.6). The minimum infective dose has not been determined, although a dose of 10^9 organisms which was orally taken by a human volunteer caused an acute enteritis within 24 hours, with symptoms lasting for four weeks [Szita *et al.* 1973].

1.6.1 Foods

Diverse food stuffs

Diverse food stuffs have been associated with yersiniosis. In the recurrent American outbreaks between 1976 and 1983, pasteurized chocolate milk [Black *et al.* 1978], reconstituted powdered milk and turkey chow mein [Shayegani *et al.* 1983], bean sprouts [Cover & Aber 1989], tofu [Aulisio *et al.* 1983] and pasteurized milk [Tacket *et al.* 1984] have been traced as vehicles of the bacterium (see the frame at the beginning of this chapter). In addition, there were links between infection and the consumption of bottled milk in outbreaks of yersiniosis in Japan [Maruyama 1987], the United Kingdom [Greenwood & Hooper 1990], Sweden [Alsterlund *et al.* 1995] and, more recently, in the USA [Shorter *et al.* 1998]. However, although raw and pasteurized milk are frequently reported to contain *Y. enterocolitica*, the isolation rates vary largely (0 to 80%) [Swaminathan *et al.* 1982; Davidson & Sprung 1989; Larkin *et al.* 1991; Rea *et al.* 1992; Desmasures *et al.* 1997] and pathogenic strains are only sporadically isolated [Adesiyun *et al.* 1996; Özbaz & Aytaç 1993]. Only in one case, an exceptionally high prevalence (15%) of pathogenic strains in raw milk has been found, i.e. in an area in the USA, but this was blamed on cross-contamination between swine and dairy animals on the farms [Rohrbach *et al.* 1992]. In contrast to their implication in various outbreaks, milk and bean products have never been associated with sporadic disease in the USA, nor in the high incidence countries in Europe [Ostroff 1995]. Worldwide, most of the isolated cases of yersiniosis are related to pork or porcine intestines [Lee *et al.* 1991; AlMohsen *et al.* 1997; Bien 1998; Tauxe *et al.* 1987; Ichinohe *et al.* 1991], like most of the recent outbreaks in the USA [Lee *et al.* 1990; DeBuono 1995].

Pork and pork products

Strong indirect evidence exist that swine (*Sus scrofa*) constitute the main reservoir for *Y. enterocolitica* strains pathogenic to humans, and that the major mode of transmission is pork. **First**, swine are frequently healthy carriers of human pathogenic strains, which live in their oral cavity or as faecal commensals. In Belgium and Norway, for example, serotypes O:3 and/or O:9 have been isolated from 50-100% of porcine tongues and/or tonsils [Wauters *et al.* 1988b; Nesbakken 1988]. Medium to high isolation rates (15-60%) of pathogenic serotypes from pig's throat and/or faeces have been reported from many other European countries [Asplund *et al.* 1990; Andersen *et al.* 1991; de Boer *et al.* 1991 1998; Bülte *et al.* 1992], and [Mousing *et al.* 1997; Atanassova & Ring 1998; Stefanov 1998], as well as from Japan [Shiozawa *et al.* 1991], and North- [Doyle *et al.* 1981; Kotula & Sharar 1993; Funk *et al.* 1998], South- [Escudero *et al.* 1996; Borie *et al.* 1997] and Central America [Adesiyun & Krishnan 1995]. In India, 2% of pigs were found to be infected with *Y. enterocolitica* O:9 [Singh *et al.* 1983]. The genetically close relationship between clinical and porcine isolates has been confirmed by means of various techniques, including total restriction endonuclease analysis, either in chromosomal DNA (REAC) [Kapperud *et al.* 1990] or in virulence plasmids (REAP) [Nesbakken *et al.* 1987; Fukushima *et al.* 1997]; analysis of restriction fragment length polymorphism in rRNA genes (RFLP-'ribotyping')[Andersen & Saunders 1990; Blumberg *et al.* 1991]; and multilocus enzyme electrophoresis (MLEE) [Caugant *et al.* 1989; Dolina & Peduzzi 1993].

Second, the naturally present bacteria may easily contaminate a carcass during the slaughtering process and increase to considerable amounts during prolonged cold storage of the raw product, a practice which is not uncommon in the meat industry [Nesbakken 1992]. Pathogenic *Y. enterocolitica* strains are indeed frequently encountered as surface contaminants in abattoirs [Andersen 1988; Nesbakken 1988], and *Y. enterocolitica* serotypes O:3 and O:9 have been found in the sludge of seven out of thirteen Dutch slaughterhouses [Fransen *et al.* 1996]. The rate of surface contamination of the raw meat could be reduced markedly by improved slaughtering techniques [Andersen 1988; Andersen *et al.* 1991; Nesbakken *et al.* 1994], but residual low levels are still found [Andersen 1998]. Furthermore, abattoirs furnish favourable conditions for psychrotrophic organisms to develop [Sammarco *et al.* 1997], as also appeared from an American study, in which pathogenic *Yersinia* could be isolated from only 0.4% of loins on the slaughter floor, but from 4.4% of vacuum-packed loins stored for 36 days at 2 °C [Saide-Albornoz *et al.* 1995].

Third, *Y. enterocolitica* serotypes O:3 and O:9 are frequently present in pork products at the retail level. In the Netherlands, for example, *Y. enterocolitica* and related species were isolated from a great variety of foods, but minced pork and porcine tonsils were found to be the only source of virulent strains [de Boer *et al* 1986,1991; de Boer 1995].

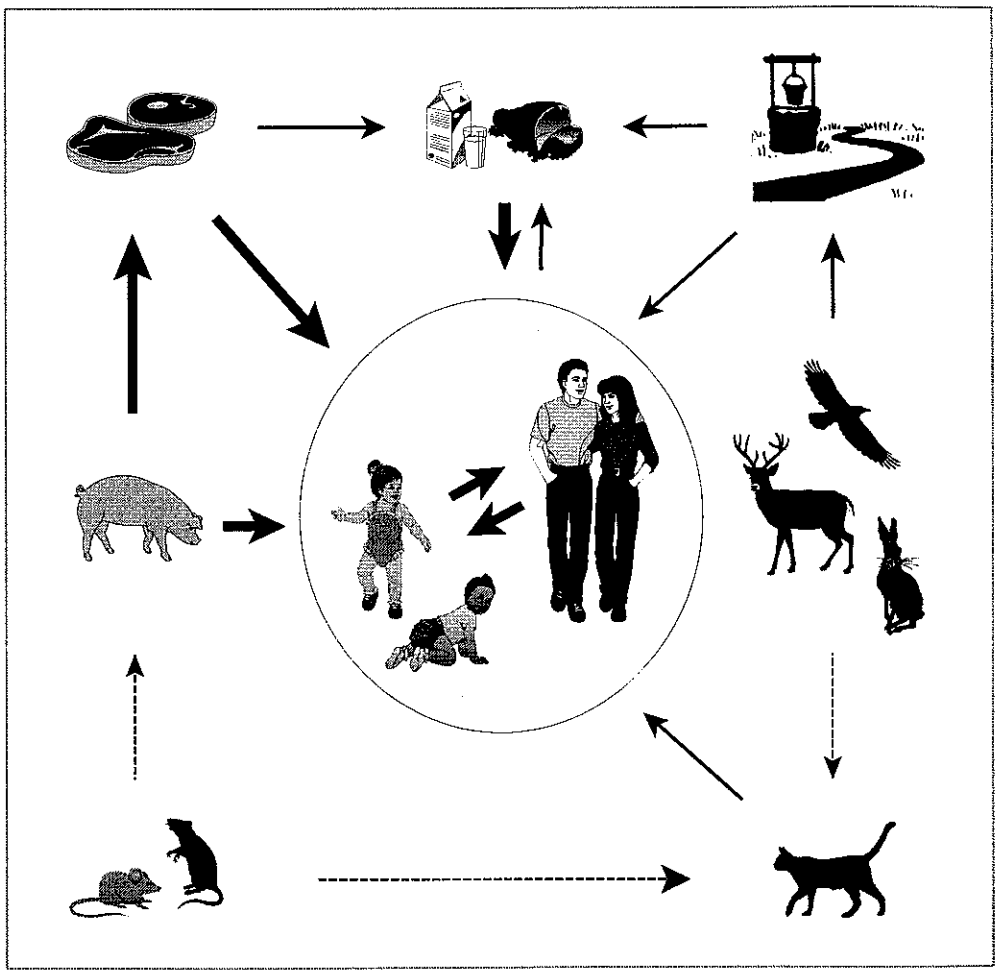


Figure 1.6 Transmission routes and recognized vehicles of *Y. enterocolitica*. The orientation and the heaviness of the arrows correspond with, respectively, the direction of the transmission and the level of documentation (heavy lines: well-documented; thin lines: rare; dotted lines: reasonable, but little or no documentation). Adapted from [Schiemann 1989].

Similarly, not inconsiderable isolation rates of pathogenic serotypes from raw pork have been obtained in many countries, including Belgium (24%) [Wauters *et al.* 1988], Japan (36%) [Shiozawa *et al.* 1987], Denmark (30%) [Andersen *et al.* 1991], Ireland (40%) [Logue *et al.* 1996], Canada (14%) [Durisin *et al.* 1997] and Spain (9%) [Garcia-Jalon *et al.* 1998]. Pathogenic strains have also been isolated from other raw meat products, such as chicken and lamb, but the prevalence

is usually much lower [Norberg 1981; Logue *et al.* 1996] and might result from cross-contamination, since meat chopping boards in butcher shops are sometimes highly contaminated with these organisms [Asakawa *et al.* 1979; Christensen 1987a].

Fourth, there is a striking relationship between the incidence of human yersiniosis and pork consumption. As early as 1973, a positive relationship was observed between the domestic slaughter of pigs, traditionally in the period December to March, and the yearly increased frequency of yersiniosis in Czechoslovakia [Rakovský *et al.* 1973]. Similarly, the first recorded case - and familial outbreak - of yersiniosis in India was directly linked to consumption of meat from a freshly slaughtered Ye O:9 infected pig [Pramanik *et al.* 1980]. Likewise, raw sausages and minced meats containing pork were identified as transmission vectors of human yersiniosis in Spain [Mendoza *et al.* 1996], and an epidemic in East-Kazakhstan was reported to result from consumption of meat from sick animals after an outbreak of yersiniosis in a pig-breeding farm [Dmitrovsky *et al.* 1998]. Furthermore, the high incidence of yersiniosis in various countries in West- and Northern Europe coincides with a high pork consumption [Kapperud *et al.* 1995], and several case-control studies and surveys in these countries have elucidated pork consumption as a risk-factor for catching yersiniosis [Ostroff *et al.* 1994]. In Moslem countries on the other hand, and among other religious groups or cultures that traditionally do not consume pork, yersiniosis is rare [Haghighi 1979; Samadi *et al.* 1982; Jegathesan *et al.* 1984].

Finally, direct contact with raw pork, especially in the setting of food preparation, was also shown to be a risk factor. In 1969, yersiniosis, although being very rare in North-Africa, was suggested as being transmitted by raw meat, since butchers appeared to be healthy carriers of the bacterium [Makulu *et al.* 1969]. More recently, some culturally determined habits, such as consumption of foods containing raw pork (a habit quite common in Belgium) or the household preparation of swine intestines (as is usual at Thanksgiving among certain populations in the USA), were shown to be highly associated with yersiniosis [Tauxe *et al.* 1987; Lee *et al.* 1990 1991; DeBuono 1995; Bien 1998]. Handling of raw pork appeared to be the ultimate cause of infection in the first reported case of yersiniosis due to serotype O:8 outside the USA [Ichinohe *et al.* 1991].

1.6.2 Drinking Water

Y. enterocolitica's wide distribution in nature and its ability to grow at low temperatures have led to the suspicion that drinking water could also be a reservoir for man. Indeed, the organism has frequently been isolated from drinking water, but in most cases only non-pathogenic variants. Nevertheless, several reports describe a coincidence between gastro-enteritis and consumption of water contaminated with *Y. enterocolitica*. In Norway, for example, a serotype O:13 strain was isolated from a human patient as well as from the well-water she had been drinking [Lassen 1972]. Similarly, an outbreak of gastrointestinal illness in a ski-resort in the USA in 1974/1975 was most likely caused by consumption of well-water contaminated with

Y. enterocolitica serotypes O:3 and O:5 [Eden *et al.* 1977]. In addition, one of the earliest cases of yersiniosis reported in the United States, due to serotype O:8, was traced to consumption of water from a mountain stream, and wild animals were suggested to have played a role in contamination of the water [Keet 1974]. It was therefore suggested that the commonly reported seasonal variation of yersiniosis outbreaks, peaking in autumn and winter, could be due to specific outgrowth of *Y. enterocolitica* in the cold season, after spread into the waterways in the summer months [Hurvell 1981]. More recently, the organism was indeed shown to be able to proliferate in water stored at 4°C [Karapinar & Gönül 1991]. In Japan, *Y. enterocolitica* serotype O:3 was isolated from surface water in the vicinity of a piggery that raised Ye O:3 positive pigs [Fukushima *et al.* 1984], and sporadic cases of human O:8 infection have been attributed to the drinking of mountain stream water that was likely contaminated with the faeces of wild rodents carrying this strain [Ohtomo *et al.* 1995]. In Italy, serotype O:7,8, which had previously been associated with gastro-intestinal infection in this country, was isolated from river water [Massa *et al.* 1988], and in Australia, pathogenic *Yersinia* were detected, by means of PCR, in an environmental water reservoir [Sandery *et al.* 1996].

Indirect consumption of environmental waters has also been implicated in yersiniosis: water used in the manufacturing process was identified as the source of outbreaks linked to the consumption tofu and bean sprouts in the USA [Tacket *et al.* 1985; Cover & Aber 1989], and water used to dilute buttermilk was the cause of the first reported foodborne outbreak of yersiniosis in India [Abraham *et al.* 1997]. Furthermore, drinking water in a medical residence was found to contain *Y. enterocolitica* after *Y. frederiksenii* had been isolated from several, ill as well as asymptomatic, members of the hospital staff [Cafferkey *et al.* 1993].

1.6.3 Live Animals

Pets

Because of their close contact with humans, pet animals have long been suspected to be a reservoir for human infections with *Y. enterocolitica*. Dogs and cats have occasionally been found to harbour pathogenic serotypes, particularly O:3, O:5,27 and O:9 [Fukushima *et al.* 1987; Chiesa *et al.* 1987; Fenwick *et al.* 1994], and outbreaks in breeding kennels as well as infection-transmission studies showed that these animals might be asymptomatic carriers [Fantasia *et al.* 1985; Fantasia *et al.* 1993; Fenwick *et al.* 1994]. Several sporadic cases and small scale outbreaks of human yersiniosis have been described in which ill dogs or cats were most likely the source of infection. On some occasions, identical pathogenic serotypes (O:20, O:3 or O:9) were isolated from ill or asymptomatic pets and human contacts [Ahvonen *et al.* 1973; Gutman *et al.* 1973; Wilson *et al.* 1976], but transmission to man could not be proven, or seemed at least doubtful. Some observations in Japan suggested involvement of wild animals in the spread of yersiniosis: the presence of *Y. enterocolitica* serotype O:8 in wild-living rodents in a restricted period coincided with the occurrence of sporadic cases of human yersiniosis, due to identical

strains, in the same period and area [Hayashidani *et al.* 1995; Ohtomo *et al.* 1995]. Similarly, wild rodents seem to play an active role in the spread of yersiniosis in Kazakhstan: peaks in human yersiniosis due to serotype O:5 (in 1992 and 1995) followed epizootic manifestations of infection with the same serotype in rats in 1991 and 1994 [Necrasova *et al.* 1998a]. In addition, a Danish study showed that wild rats and mice living on swine-breeding farms are potential reservoirs of *Y. enterocolitica* O:3, too [Bech-Nielsen *et al.* 1998]. Indirect support for possible spread through wild animals also comes from the observed carriage of serotypes O:5,27 and O:3 by hares in Germany [Wuthe *et al.* 1995], but a possible relation with human yersiniosis has not been investigated.

Farm animals

Suspicion has also been directed at contact with domestic animals in the food industry, especially swine, a common risk for farm- or abattoir-workers. After a labourer on a pig farm in South Africa died of septicemia due to *Y. enterocolitica* O:3, animals from this farm were inspected, and the identical strain was found in rectal swabs of 50% of the pigs [Rabson & Koornhof 1973]. At the same time, increased anti-*Y. enterocolitica* titres were found among healthy pig-slaughterers, suggesting past contact with the organism and occupational risk from exposure to pigs. The involvement of swine in putting humans in contact with this bacterium is also supported by data obtained from serological studies in Scandinavia and Italy [Merilahti-Palo *et al.* 1991; Seuri & Granfors 1992; Franzin & Curti 1993; Nesbakken *et al.* 1991; Franzin *et al.* 1998].

1.6.4 Humans

Epidemiological studies concerning many small scale familial outbreaks of yersiniosis suggest that the disease can easily be spread in contacts with infected people, possibly via highly contaminated stool [Ahvonen & Rossi 1970; Gutman *et al.* 1973; Szita *et al.* 1973; Marks *et al.* 1980; Martin *et al.* 1982]. Communicability of the illness is also evidenced by the large scale outbreaks that occurred among school populations [Asakawa *et al.* 1973; Olšovský *et al.* 1975Kasatiya 1976], as well as by several hospital-acquired episodes, affecting both in-patients, staff and their relatives [Toivanen *et al.* 1973; Ratnam *et al.* 1982; McIntyre & Nnochiri 1986; Cannon & Linnemann 1992].

1.6.5 Blood-transfusions

Even more worrisome than the direct transmission between humans, is the indirect person-to-person transmission of *Y. enterocolitica* which may occur during blood transfusions. This is a rare but easily fatal complication arising from contaminated blood products, especially red blood cell concentrates [Prentice 1992]. The rate of *Yersinia*-associated adverse transfusion reactions has been estimated to lie between 0.1 and 2 in one million transfusions [Högman & Engstrand 1996; Halpin *et al.* 1997]. As is to be expected, this type of infection does not result in gastro-enteritis; it usually leads to septic shock, a severe disorder with a high mortality rate.

Since the first published case of *Y. enterocolitica* sepsis, which occurred in the Netherlands in 1975 [Bruining & De Wilde-Beekhuizen 1975], more than 50 cases of transfusion reaction caused by this organism - with a mortality rate higher than 60% - have now been described in the English and German literature [Högman & Engstrand 1996; Höher 1996]. In addition to the common pathogens O:3, O:9 and O:5,27, which were causative agents in most cases of transfusion-acquired *Yersinia* infection with fatal outcome [Högman & Engstrand 1996; Neumeister *et al.* 1997; Theakston *et al.* 1997; Mewis *et al.* 1997], more rare serotypes as O:20 and O:1,2,3 have also been involved [Tipple *et al.* 1990]. In several transfusion-acquired cases, stool culture and/or serology revealed overt *Yersinia* infections in the blood donor [Bjune *et al.* 1984; Brown & White 1988; Haverly *et al.* 1996; Kuehnert *et al.* 1997] or asymptomatic carriage of the bacterium [Stenhouse *et al.* 1982; Brown & White 1988; Elrick 1988; McDonald *et al.* 1996]. In other cases, however, donors were negative for *Y. enterocolitica* in blood and stool cultures, as well as in antibody-serology [Neumeister *et al.* 1997]. The opposite result has also been observed: *Y. enterocolitica* serotype O:3 was cultured from blood containing a high antibody titre against Ye O:3, donated by a donor with a history of surgically treated pseudo-appendicitis, but the recipient of the erythrocyte concentrate did not experience any adverse effects from the transfusion [Jacobs *et al.* 1989]. Nevertheless, most reports of transfusion-acquired septicemia include serological evidence of donor infection with *Y. enterocolitica*, with or without a history of diarrhoea at the time of donation [CDC 1991; Högman & Engstrand 1996]. Thus, there is strong evidence for a link between the increase of transfusion-associated fatalities and an increased incidence of *Y. enterocolitica* gastroenteritis [CDC 1991]. This suggestion was recently supported by a report from New Zealand in which a high incidence of transfusion-transmitted *Y. enterocolitica* infections has been correlated with a rise in the number of faecal isolations [Theakston *et al.* 1997].

1.7 INFLUENCE OF COLD STORAGE

Effects in foods

A number of studies have shown that *Y. enterocolitica* is able to multiply in many types of refrigerated foods, especially raw and cooked meat [Hanna *et al.* 1977; García de Fernando *et al.* 1995; Greer *et al.* 1995; Shenoy & Murano 1996], but also in dry sausages [Kleemann & Bergann 1996]; in dairy products, such as milk [Stern *et al.* 1980a,1980b; Kendall & Gilbert 1980], butter [Slavchev 1989; Lanciotti *et al.* 1992], and wet, soft as well as dry cheeses [Sims *et al.* 1989; Little & Knochel 1994; Sarigiannidou *et al.* 1997]; in fresh and boiled eggs [Erickson 1995]; in boiled rice and potatoes [Kendall & Gilbert 1980]; in raw and smoked fish [Hudson & Mott 1993; Davies & Slade 1995]; and in shell-fish [Hudson & Avery 1994]. It has also been reported that only serotypes O:3, O:8 and O:9 grow well at 4 °C, while other serotypes did not [Eiss 1975]. Freezing conditions may have an adverse effect on the survival of the bacterium [Hanna *et al.* 1977; Asakawa *et al.* 1979], but freezing tolerance has also been reported [Leistner *et al.* 1975; Kendall & Gilbert 1980; Slavchev 1989]. On the other hand, the

ability of *Y. enterocolitica* to compete with other psychrotrophic organisms normally present in food may be poor [Fukushima & Gomyoda 1986; Kleinlein & Untermann 1990], and growth may also be inhibited by components of the product itself, such as minced meat exudates [Doherty *et al.* 1995]. At retail level, pathogenic serotypes have been found in various refrigerated foods of animal origin, including cooked meat products and fish fillets [Velázquez *et al.* 1993; Velázquez *et al.* 1996]. In addition, significantly higher amounts of pathogenic strains were found, in Taiwan, in refrigerated or frozen stored pork products compared to non-refrigerated products [Tsai & Chen 1991]. These observations indicate that cold proliferation of the bacterium is not just a laboratory bound-phenomenon.

Effects in blood products

From retrospective investigations in cases of transfusion-acquired *Yersinia* infections, it was concluded that extended storage at low temperature might have a disastrous influence on the quality of blood products, and llaboratory studies support this conclusion. In almost all cases of transfusion-acquired septicemia, the donated whole blood or red cell concentrates had been stored for over three weeks before transfusion, and a number of laboratory studies have shown that under conditions of cold storage and iron enrichment from aging erythrocytes, *Y. enterocolitica* can proliferate after a lag phase of 10-20 days [Arduino *et al.* 1989; Franzin & Gioannini 1992]. Obviously, those serotypes with a high iron requirement, i.e. those which do not produce endogeneous siderophores, will benefit most from the iron overload. The difference in relative advantage from iron enrichment, in combination with a difference in serum resistance, might explain why serotype O:8 has never been found in cases of transfusion-associated bacteraemia, while serotype O:3 is the most common cause of this type of *Y. enterocolitica* infection [Neumeister *et al.* 1997]. It has also been suggested that, during temperature fall after blood donation, the organism might utilize its invasive capacity by expressing the *inv* gene, to reach a protective side inside a cell. Resistance to intracellular killing mechanisms is low at 37°C but increases with decreasing temperatures, which might explain why pre-storage leucocyte depletion has a protective effect on contamination with the bacterium during prolonged storage of blood products [Högman & Engstrand 1996; Neumeister *et al.* 1997].

1.8 ISOLATION & IDENTIFICATION

1.8.1 Selective Media

Y. enterocolitica strains show a marked degree of variability in ability to grow and appearance on commonly used enteric isolation media. On such media, the usually very small and unspecific colonies can easily be overlooked, especially when surrounded by an abundant growth of heterogenous native flora. This has possibly accounted for its initially delayed

recognition and has led to the development of several specific media and enrichment techniques [Pee and Stragier 1979; Head *et al.* 1982]. For isolation of pathogenic strains from the heavily contaminated faecal samples of symptomatic persons, direct plating on specific media such as Cefsoludin-Irgasan-Novobiocin (CIN) and Desoxycholat agar is satisfactory [Wauters 1973; Butler 1981]. For the recovery of *Y. enterocolitica* from less contaminated samples such as foods, cold preincubation, specific enrichments, e.g. with Irgasan-ticarcillin-potassium chlorate (ITC), and polycarbonate surface adhesion have proven to be valuable techniques [Wauters *et al.* 1988b; Sheridan *et al.* 1998]. However, polycarbonate surface adhesion is non-selective and cold enrichment also favours growth of the non-pathogenic strains, so these isolation techniques do not take away the need of methods to differentiate between pathogens and non-pathogens.

1.8.2 Classical Virulence Tests

The differentiation between virulent and non-virulent strains has, until the onset of the 1990s, mainly relied upon bio- and serotyping, based on the strong association of pathogenic significance with only a few serogroup-biovar combinations. In addition, a number of different in-vitro and in-vivo tests have been used to distinguish between pathogens and non-pathogens. These tests include various animal models, using either gerbils, mice, rabbits or guinea pigs [reviewed by Carter 1981 and Schiemann 1989]; in-vitro invasiveness [Une 1977; Prpic *et al.* 1985]; resistance to the bactericidal action of blood serum [Pai & DeStephano 1982]; autoagglutination and calcium-dependent growth at 37°C [Laird & Cavanaugh 1980; Gemski *et al.* 1980]; binding of dyes like Congo red or Crystal violet [Prpic *et al.* 1983; Dziezak 1991]; and pyrazinamidase-activity [Kandolo & Wauters 1985]. However, many of these tests are based on properties associated with the virulence plasmid, and/or are subject to problems of gene expression in vitro, which make their sensitivity and selectivity questionable [Wachsmuth *et al.* 1984; Farmer *et al.* 1992].

1.8.3 DNA-based Methods

Several molecular genetic typing methods have been used for detection and/or identification of virulent strains. Applying DNA-hybridizations, the presence of pYV could easily be detected, independent of gene-expression [Hill *et al.* 1983; Jagow & Hill 1986], and after 1989, when the polymerase chain reaction (PCR) technique appeared on the scene, much progress was gained in sensitivity and specificity of the pYV-detection methods [Miliotis *et al.* 1989; Wren & Tabaqchali 1990; Kapperud *et al.* 1990, Nesbakken *et al.* 1991]. However, neither of these pYV-targeted methods overcame the problem of false negative results that might occur due to the easy loss of the virulence plasmid during the isolation procedure. An escape from this dilemma was found in the development of DNA-based methods that target chromosomal virulence associated sequences: distinguishing virulent and non-virulent strains was shown to be possible

et al. 1992] or *yst* [Delor *et al.* 1990]. These probes have successfully been applied to detect pathogenic strains in clinical specimens, raw meat samples, foods and environmental waters [Ibrahim *et al.* 1992a 1992b 1997; Harnett *et al.* 1996; Thisted Lambertz *et al.* 1996; Sandery *et al.* 1996]. Excellent results in detection, as well as in distinguishing pathogenic and non-pathogenic strains, have also been obtained with DNA-probe- and/or PCR-based tests that simultaneously target both chromosomal and plasmid-borne virulence genes [Nakajima *et al.* 1992; Thisted Lambertz *et al.* 1996; Fliss *et al.* 1995; Harnett *et al.* 1996; Weynants *et al.* 1996; Nilsson *et al.* 1998]. In addition, genetic relatedness among strains, a tool useful in epidemiological studies, has successfully been studied by combining various DNA-based methods, including whole-cell DNA RFLP analysis by hybridizations with rRNA-based probes ('ribotyping') [Iteman *et al.* 1996; Lobato *et al.* 1998]; random amplified polymorphic DNA (RAPD)-fingerprinting [Odinot *et al.* 1995]; and pulsed-field gelelectrophoresis (PFGE) analysis of restriction endonuclease profiles obtained from plasmid (REAP) or chromosomal (REAC) DNA [Buchrieser *et al.* 1994; Najdenski *et al.* 1994; Asplund *et al.* 1998].

Good results in detection of (antigens from) virulent strains has also been obtained by applying specific immunofluorescent antibodies [Viitanen *et al.* 1991, Sheridan *et al.* 1998] and by in-situ hybridizations with fluorescently tagged oligonucleotide probes [Trebesius *et al.* 1998].

REFERENCES

Books, Theses and Reviews

1. **Asplund K.** 1998. *Epidemiological and ecological aspects of Yersinia enterocolitica serotype O:3.* PhD Thesis, University of Helsinki, Finland.
2. **Bliska JB.** 1994. Yops of the pathogenic *Yersinia* spp. In: *Molecular genetics of bacterial pathogenesis.* Miller VL, Kaper JB, Portnoy DA, and Isberg RR (eds). ASM Press, Washington, USA, pp 365-381.
3. **Bottone EJ.** 1977. *Yersinia enterocolitica*: a panoramic view of a charismatic microorganism. *Crit Rev Microbiol* **5**: 211-241.
4. **Bottone EJ (ed).** 1981. *Yersinia enterocolitica* CRC Press, Boca Raton, Florida, USA.
5. **Bottone EJ.** 1997. *Yersinia enterocolitica*: the charisma continues. *Clin Microbiol Rev* **10**: 257-276.
6. **Butler T.** 1983. Plague and other *Yersinia* infections. *Curr Top Infect Dis.* Plenum Press, New York, USA.
7. **Carter PB, Lafleur L, and Toma S (eds).** 1979. *Yersinia enterocolitica*: biology, epidemiology, and pathology. *Contr Microbiol Immunol*, **Vol 5**, Karger, Basel, Switzerland.
8. **Cornelis GR.** 1994. *Yersinia* pathogenicity factors. *Curr Top Microbiol Immunol* **192**: 243-263.
9. **Cornelis G, Laroche Y, Balligand G, Sory MP, Wauters G.** 1987. *Yersinia enterocolitica*, a primary model for bacterial invasiveness. *Rev Inf Dis* **9**: 64-87.
10. **Cornelis GR, Biot T, Lambert de Rouvroit C, Michiels T, Mulder B, Sluiters C, Sory MP, Van Bouchaute M, and Vanooteghem JC.** 1989. The *Yersinia yop* regulon. *Mol Microbiol* **3**: 1455-1459.
11. **Cover TL, and Aber RC.** 1989. *Yersinia enterocolitica. N Engl J Med* **321**: 16-24.
12. **Högman CF, and Engstrand L.** 1996. Factors affecting growth of *Yersinia enterocolitica* in

cellular blood products. *Transfus Med Rev* **10**: 259-275.
13. **Höher PG.** 1996. Mikrobielle Sicherheit von Blutprodukten. *Infusionsther Transfusionsmed* **23**: 42-58.
14. **Nesbakken T.** 1992. *Epidemiological and food hygienic aspects of Yersinia enterocolitica with special reference to the pig as a suspected source of infection.* PhD Thesis, Norwegian college of Veterinary Medicine, Oslo, Norway.
15. **Pierson DE.** 1994. Mechanisms of *Yersinia* entry into mammalian cells. In: *Molecular genetics of bacterial pathogenesis.* Miller VL, Kaper JB, Portnoy DA, and Isberg RR (eds). ASM Press, Washington, USA, pp 235-247.
16. **Portnoy DA, and Martinez RJ.** 1985. Role of a plasmid in the pathogenicity of *Yersinia* species. *Curr Topics Microbiol Immunol* **118**: 29-51.
17. **Prpic JK, and Davey RB (eds).** 1987. The genus *Yersinia*: epidemiology, molecular microbiology and pathogenesis. *Contr Microbiol Immunol*, **Vol 9**, Karger, Basel, Switzerland.
18. **Ravagnan G, and Chiesa C (eds).** 1995. Yersiniosis: present and future. *Contr Microbiol Immunol*, **Vol 13**, Karger, Basel, Switzerland.
19. **Schiemann DA.** 1989. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. In: *Foodborne bacterial pathogens*. Doyle MP (ed), Marcel Dekker Inc, New York, USA, pp 601-672.
20. **Stern NJ, and Pierson MD.** 1979. *Yersinia enterocolitica*: a review of the psychrotrophic water and foodborne pathogen. *J Food Sci* **44**: 1736-1742.
21. **Swaminathan B, Harmon MC, and Mehlman IJ.** 1982. A review: *Yersinia enterocolitica*. *J Appl Bacteriol* **52**: 151-183.
22. **Une T, Maruyama T, and Tsubokura M (eds).** 1991. Current investigations of the microbiology of Yersiniae. *Contr Microbiol Immunol*, **Vol 12**, Karger, Basel, Switzerland.
23. **Wauters G.** 1970. *Contribution a l'étude de Yersinia enterocolitica*. PhD Thesis, Université Catholique de Louvain, Vander, Bruxelles, Belgium
24. **Winblad S (ed).** 1973. Yersinia, Pasteurella and Francisella. *Contr Microbiol Immunol*, **Vol 2**, Karger, Basel, Switzerland.

Other References

25. **Abbas CA, and Card GL.** 1980. The relationships between growth temperature, fatty acid composition and the physical state and fluidity of membrane lipids in *Yersinia enterocolitica*. *Biochim Biophys Acta* **602**: 469-476.
26. **Abraham M, Pai M, Kang G, Asokan GV, Magesh SR, Bhattacharji S, and Ramakrishna BS.** 1997 An outbreak of food poisoning in Tamil Nadu associated with *Yersinia enterocolitica*. *Indian J Med Res* **106**: 465-468.
27. **Adamkiewicz TV, Berkovitch M, Krishnan C, Polsinelli C, Kermack D, and Olivieri NF.** 1998. Infection due to *Yersinia enterocolitica* in a series of patients with beta-thalassaemia: incidence and predisposing factors. *Clin Infect Dis* **27**: 1362-1366.
28. **Adams MR, and Moss MO.** 1995. *Food Microbiology.* The Royal Society of Chemistry, Cambridge, UK.
29. **Adesiyun AA, and Krishnan C.** 1995. Occurrence of *Yersinia enterocolitica* O:3, *Listeria monocytogenes* O:4 and thermophilic *Campylobacter* spp. in slaughter pigs and carcasses in Trinidad. *Food Microbiol* **12**: 99-107.
30. **Adesiyun AA, Webb LA, Romain H, and Kaminjolo JS.** 1996. Prevalence of *Salmonella, Listeria monocytogenes, Campylobacter* spp., *Yersinia enterocolitica* and *Cryptosporidium* spp. in bulk milk, cows' faeces and effluents of dairy farms in Trinidad. *Revue Élev Méd Vét Pays Trop* **49**: 303-309.
31. **Ahvonen P, and Rossi T.** 1970. Familial occurrence of *Yersinia enterocolitica* infection and acute arthritis. *Acta Paediatr Scand Suppl* **206**: 121-122.
32. **Ahvonen P.** 1973. *Yersinia enterocolitica* infections in Finland. *Contr Microbiol Immunol* **2**: 133-134.

33. **Ahvonen P, Thal E, and Vasenius H.** 1973. Occurrence of *Yersinia enterocolitica* in animals in Finland and Sweden. *Contr Microbiol Immunol* **2**: 135-136.
34. **Akkermans JPWM, and Terpstra JI.** 1963. Pseudotuberculose bij chinchilla's veroorzaakt door een bijzondere species (In Dutch) <Pseudotuberculosis in chinchillas caused by a special species.> *Tijdschr Diergeneesk* **88**: 91-95.
35. **Albert G, and Lafleur L.** 1971. *Yersinia enterocolitica* in children. *Can J Public Health* **62**: 70-71.
36. **Aldová E, and Svandova E.** 1984. *Yersinia enterocolitica* O:3 findings in porcine tongues in comparison with yersiniosis incidence in Czechoslovakia. *J Hyg Epidemiol Immunol* **28**: 319-329.
37. **Aleksić S, Steigerwalt AG, Bockemühl J, Huntley-Carter GP, and Brenner DJ.** 1987. *Yersinia rohdei* sp. nov. isolated from human and dog faeces and surface water. *Int J Syst Bact* **37**: 327-332.
38. **Aleksić S, and Bockemühl J.** 1990 Mikrobiologie und Epidemiologie der Yersiniosen. *Immun Infekt* **18**: 178-185.
39. **Aleksić S.** 1995. Occurrence of *Y. enterocolitica* antigens O:3, O:9 and O:8 in different *Yersinia* species, their corresponding H-antigens and origin. *Contr Microbiol Immunol* **13**: 89-92.
40. **Aleksić S, Bockemühl J, and Wuthe HH.** 1995. Epidemiology of *Yersinia pseudotuberculosis* in Germany 1983-1993. *Contr Microbiol Immunol* **13**: 55-58.
41. **Aleksić S, and Bockemühl J.** 1996. Untersuchungen von Yersinia-Stämmen aus Deutschland 1993-1994. *Bundesgesundheitsbl* **G96-136**: 94-97.
42. **AlMohsen I, Luedtke G, and English BK.** 1997. Invasive infections caused by *Yersinia enterocolitica* in infants. *Pediatr Infect Dis J* **16**: 253-255.
43. **Alonso JM, Bercovier H, Servan J, Bourdin M, and Mollaret HH.** 1976. Contribution à l'étude épidémiologique des infections à *Yersinia enterocolitica*. II. Enquête écologique. *Méd Mal Infect* **6**: 434-441.
44. **Alonso JM, Bercovier H, Servan J, and Mollaret HH.** 1979. Contribution to the study of the ecology of *Yersinia enterocolitica* in France. *Contr Microbiol Immunol* **5**: 132-143.
45. **Alsterlund R, Danielsson-Tham ML, Karp G, Eden T, Jong B de, Nilsson PO, and Ransjö U.** 1995. *Yersinia enterocolitica* -utbrott på Bjärehalvön; visar på risker med kylda matvaror. (In Swedish). <An outbreak of *Yersinia enterocolitica* infection on the Bjaerred peninsula; indications for risks of refrigerated food.> *Lakartidningen* **92**: 1213-1214.
46. **Andersen JK.** 1988. Contamination of freshly slaughtered pig carcasses with human pathogenic *Yersinia enterocolitica*. *Int J Food Microbiol* **7**: 193-202.
47. **Andersen JK.** 1998. *Yersinia enterocolitica* in the retail sector in Denmark. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Suppl II* **6**: S48.
48. **Andersen JK, and Saunders NA.** 1990. Epidemiological typing of *Yersinia enterocolitica* by analysis of restriction fragment length polymorphisms with a cloned ribosomal RNA gene. *J Med Microbiol* **32**: 179-187.
49. **Andersen JK, Sørensen R, and Glensbjerg M.** 1991. Aspects of the epidemiology of *Yersinia enterocolitica*: a review. *Int J Food Microbiol* **13**: 231-238.
50. **Anonymus.** 1987. (In Chinese). <First report of an outbreak caused by *Yersinia enterocolitica* serotype O:9 in Shengyang.> *Chin J Epidemiol* **8**: 264-267.
51. **Anyanwu BN.** 1995. *Yersinia enterocolitica*: its importance in Nigeria's health management. *Int J Environ Health Res* **5**: 87-92.
52. **Arduino MJ, Bland LA, Tipple MA, Aguero SM, Favero MS, and Jarvis WR.** 1989. Growth and endotoxin production of *Yersinia enterocolitica* and *Enterobacter agglomerans* in packed erythrocytes. *J Clin Microbiol* **27**: 1483-1485.
53. **Asakawa Y, Akahane S, Kagata N, Noguchi M, Sakazaki R, and Tamura K.** 1973. Two community outbreaks of human infection with *Yersinia enterocolitica*. *J Hyg Camb* **71**: 715-723.
54. **Asakawa Y, Akahane S, Shiozawa K, and Honma T.** 1979. Investigations of source and route of *Yersinia enterocolitica* infection. *Contr Microbiol Immunol* **5**: 115-121.
55. **Asplund K, Tuovinen V, Veijalainen P, and Hirn J.** 1990. The prevalence of *Yersinia enterocolitica* in Finnish pigs and pork. *Acta Vet Scand* **31**: 39-43.
56. **Asplund K, Johansson T, and Siitonen A.** 1998. Evaluation of pulsed-field gel electrophoresis of

genomic restriction fragments in the discrimination of *Yersinia enterocolitica* O:3. *Epidemiol Infect* **121**: 579-586.

57. **Atanassova V, and Ring C.** 1998. Occurrence of *Yersinia enterocolitica* in slaughter pigs. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S46.
58. **Attwood SEA, Cafferkey MT, West AB, Healy E, Mealy K, Buckley TF, Boyle N, and Keane FBV.** 1987. Yersinia infection and acute abdominal pain. *Lancet i*: 529-533.
59. **Aulisio CCG, Stanfield JT, Weagant SD, and Hill WE.** 1983. Yersiniosis associated with tofu consumption: serological, biochemical and pathogenicity studies of *Yersinia enterocolitica* isolates. *J Food Prot* **46**: 266-230.
60. **BAG** (Bundesamt für Gesundheit) 1993. Infektionskrankheiten. In: *BAG-bulletin* (weekly summary of notifiable diseases in Switzerland) **52**: 911.
61. **BAG** (Bundesamt für Gesundheit) 1998. In: *BAG-bulletin* (weekly summary of notifiable diseases in Switzerland) **98-1/2**: 5.
62. **Baier R, and Puppel H.** 1975. Isolierung von *Yersinia enterocolitica* bei akuter Ileites. *Med Klin* **70**: 1250-1253.
63. **Baxter E, Rose PD, and Kirby R.** 1994. Age and population group related distribution of enteropathogens in the Eastern Cape, South Africa. *Lett Appl Microbiol* **19**: 442-445.
64. **Bean NH, and Griffin PM.** 1990. Foodborne disease outbreaks in the United States 1973-1987: pathogens, vehicles, and trends. *J Food Prot* **53**: 804-817.
65. **Bech-Nielsen S, Wingstrand A, Nielsen B, Dahl J, and Lind P.** 1998. Detection of *Yersinia enterocolitica* O:3 biotype 4 in rats, mice, cats, dogs as well as from water and feed sources on swine farms. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S48.
66. **Bennett JV, Holmberg SD, Rogers MF, and Solomon SL.** 1987. Infectious and parasitic diseases. In: *Closing the gap - the burden of unnecessary illness.* Amler RW, and Dull HB (eds), Oxford Univ Press, Oxford, UK, pp 102-114.
67. **Benoit C, Guiyoule A, and Carniel E.** 1996. Sérodiagnostic des infections humaines à *Yersinia* pathogènes. *Presse Méd* **25**: 1627-1630.
68. **Bercovier H, Brenner DJ, Ursing J, Steigerwalt AG, Fanning GR, Alonso JM, Carter GP, and Mollaret HH.** 1980a. Characterization of *Yersinia enterocolitica* sensu stricto. *Curr Microbiol* **4**: 201-206.
69. **Bercovier H, and Mollaret HH.** 1984. Genus XIV *Yersinia* In:*Bergey's manual of systematic bacteriology*. Krieg NR, and Holt JG (eds), Williams & Wilkins, Baltimore, USA, vol 1: pp 498-506.
70. **Bercovier H, Steigerwalt AG, Guiyoule A, Huntley-Carter GP, and Brenner DJ.** 1984. *Yersinia aldovae* (formerly *Yersinia enterocolitica*-like group X2): a new species of *Enterobacteriaceae* isolated from aquatic ecosytems. *Int J Syst Bact* **34**: 166-172.
71. **Bercovier H, Ursing J, Brenner DJ, Steigerwalt AG, Fanning GR, Carter GP, and Mollaret HH.** 1980b. *Yersinia kristensenii*: A new species of *Enterobacteriaceae* composed of sucrose-negative strains (formerly called atypical *Yersinia enterocolitica* or *Yersinia enterocolitica*-like) *Curr Microbiol* **4**: 219-224.
72. **Bergann T, Kleemann J, and Sohr D.** 1995. Modelluntersuchungen zur Psychrotrophie von *Yersinia enterocolitica*. *J Vet Med B* **42**: 523-531.
73. **Bien JP.** 1998. Anticipatory guidance and chitterlings. *J Pediatr* **133**: 712.
74. **Bin-Sagheer, Myers J, Lapham C, and Sarubbi FA.** 1997. Meningitis caused by *Yersinia enterocolitica* case report and review. *Inf Dis Clin Prac* **6**: 198-200.
75. **Bissett ML, Powers C, Abbott SL, and Janda JM.** 1990. Epidemiologic investigations of *Yersinia enterocolitica* and related species: sources, frequency and serogroup distribution. *J Clin Microbiol* **28**: 910-912.
76. **Bjune G, Ruud TE, and Eng J.** 1984. Bacterial shock due to transfusion with *Yersinia enterocolitica* infected blood. *Scand J Infect Dis* **16**: 411-412.
77. **Black RE, Jackson RJ, Tsai T, Medvesky M, Shayegani M, Feeley JC, MacLeod KIE, and Wakelee AM.** 1978. Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. *N Engl*

J Med **298**: 76-79.

78. **Bliska JB, and Falkow S.** 1992. Bacterial resistance to complement killing mediated by the Ail protein of *Yersinia enterocolitica*. *Proc Natl Acad Sci USA* **89**: 3561-3565.
79. **Blumberg, HM, Kiehlbauch JA, and Wachsmuth IK.** 1991. Molecular epidemiology of *Yersinia enterocolitica* O:3 infections: use of chromosomal DNA restriction fragment length polymorphisms of rRNA genes. *J Clin Microbiol* **29**: 2368-2374.
80. **Bockemühl J, Schmitt H, and Bednarak I.** 1978. Zur Häufigkeit von Darmerkrankungen durch *Yersinia enterocolitica*. *Zbl Bakt Hyg I Abt Orig A* **242**: 42-51.
81. **Boer E de.** 1995. Isolation of *Yersinia enterocolitica* from foods. *Contr Microbiol Immunol* **13**: 71-73.
82. **Boer E de, Seldam WM, and Oosterom J.** 1986. Characterization of *Yersinia enterocolitica* and related species from foods and porcine tonsils in the Netherlands. *Int J Food Microbiol* **3**: 217-224.
83. **Boer E de, Nouws JFM, Nijland E, and Smulders AHJW.** 1991. Varkens(vlees) als bron van pathogene *Yersinia enterocolitica* (In Dutch). <Pigs intended for slaughter and pork as sources for pathogenic *Yersinia enterocolitica*.> *Tijdschr Diergeneesk* **116**: 277-280.
84. **Boer E de, Hulleman A, and Kleverwal M.** 1998. Comparison of culture media for the isolation of *Yersinia enterocolitica* from porcine faeces and tonsils. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S46-47.
85. **Boland A, Sory MP, Iriarte M, Kerbourch C, Wattiau, and Cornelis GR.** 1996. Status of YopM and YopN in the Yersinia Yop virulon: YopM of *Yersinia enterocolitica* is internalized inside the cytosol of PU5-1.8 macrophages by the YopB, D, N delivery apparatus. *EMBO J* **15**: 5191-5201.
86. **Borch E, Nesbakken T, and Christensen H.** 1996. Hazard identification in swine slaughter with respect to foodborne bacteria. *Int J Food Microbiol* **30**: 9-25.
87. **Borie CF, Jara MA, Sánchez ML, San Martín B, Arellano C, Martínez J, and Prado V.** 1997. Isolation and characterization of *Yersinia enterocolitica* from swine and cattle in Chile. *J Vet Med B* **44**: 347-354.
88. **Bottone EJ.** 1983. Current trends of *Yersinia enterocolitica* isolates in the New York City area. *J Clin Microbiol* **17**: 63-67.
89. **Bottone EJ, and Sheehan DJ.** 1983. *Yersinia enterocolitica*: guidelines for serologic diagnosis of human infections. *Rev Infect Dis* **5**: 898-906.
90. **Bottone EJ, Gulians CR, and Sierra MF.** 1987. Disease spectrum of *Yersinia enterocolitica* serogroup O:3, the predominant cause of human infection in New York City. *Contr Microbiol Immunol* **9**: 56-60.
91. **Boyce JM, Evans DJ, Evans DG, and DuPont HL.** 1979. Production of heat-stable, methanol-soluble enterotoxin by *Yersinia enterocolitica*. *Infect Immun* **25**: 532-537.
92. **Bradley RM, Gander RM, Patel SK, and Kaplan HS.** 1997. Inhibitory effect of 0°C storage on the proliferation of *Yersinia enterocolitica* in donated blood. *Transfusion* **37**: 691-695.
93. **Brenner DJ. 1979.** Speciation in *Yersinia*. *Contr Microbiol Immunol* **5**: 33-43.
94. **Brenner DJ, Steigerwalt AG, Falcão DP, Weaver RE, and Fanning GR.** 1976. Characterization of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* by deoxyribonucleic acid hybridization and by biochemical reactions. *Int J Syst Bacteriol* **26**:180-194.
95. **Brenner DJ, Ursing J, Bercovier H, Steigerwalt AG, Fanning GR, Alonso JM, and Mollaret HH.** 1980a. Deoxyribonucleic acid relatedness in *Yersinia enterocolitica* and *Yersinia enterocolitica*-like organisms. *Curr Microbiol* **4**: 195-200.
96. **Brenner DJ, Bercovier H, Ursing J, Alonso JM, Steigerwalt AG, Fanning GR, Carter GP, and Mollaret HH.** 1980b. *Yersinia intermedia*: A new species of *Enterobacteriaceae* composed of rhamnose-positive, melibiose-positive, raffinose-positive strains (formerly called *Yersinia enterocolitica* or *Yersinia enterocolitica*-like) *Curr Microbiol* **4**: 207-212.
97. **Brocklehurst TF, and Lund BM.** 1990. The influence of pH, temperature and organic acids on the initiation of growth of *Yersinia enterocolitica*. *J Appl Bacteriol* **69**: 390-397.
98. **Brown SE, and White SE.** 1988. *Yersinia enterocolitica* and transfusion-induced septicemia. *Anesth Analg* **67**: 415-417.

99. **Bruining M, and De Wilde-Beekhuizen CCM.** 1975. A case of contamination of donor blood by *Yersinia enterocolitica* type 9. *Medikon Ned* **4**(1): 25-26.
100. **Bucci G, Maini P, Pacifico L, Volterra L, and Chiesa C.** 1991. Direct isolation of *Yersinia enterocolitica* from stool specimens of patients with intestinal disorders. *Contr Microbiol Immunol* **12**: 50-55.
101. **Buchrieser C, Weagant SD, and Kaspar CW.** 1994. Molecular characterization of *Yersinia enterocolitica* by pulsed-field electrophoresis and hybridization of DNA fragments to *ail* and pYV probes. *Appl Environ Microbiol* **60**: 4371-4379.
102. **Buckeridge SA, Seaman A, and Woodbine M.** 1980. Effects of temperature and carbohydrate on the growth and survival of *Yersinia enterocolitica*. In: *Microbial growth and survival in extremes of environment.* Gould GW, and Corry JEL (eds). Soc Appl Bacteriol Techn Ser, Academic Press, London, UK. Vol 15: 205-214.
103. **Bülte M, Klein G, and Reuter G.** 1992 Pig slaughter. Is the meat contaminated by *Yersinia enterocolitica* pathogenic to man? *Fleischwirts* **72**: 1267-1270.
104. **Burnens AP, Frey A, and Nicolet J.** 1996. Association between clinical presentation, biogroups and virulence attributes of *Yersinia enterocolitica* strains in human diarrhoeal disease. *Epidemiol Infect* **116**: 27-34.
105. **Butler T, Carniel E, Hossain S, Alam N, and Mazigh D.** 1987. Diarrhoea caused by *Yersinia enterocolitica* in children in Bangladesh. *Contr Microbiol Immunol* **9**: 117-121.
106. **Cafferkey MT, and Buckley TF.** 1987. Comparison of saline agglutination, antibody to human gammaglobulin, and immunofluorescence tests in the routine serological diagnosis of yersiniosis. *J Infect Dis* **156**: 845-848.
107. **Cafferkey MT, Sloane A, McCrae S, and O'Morain CA.** 1993. *Yersinia frederiksenii* infection and colonization in hospital staff. *J Hosp Infect* **24**: 109-115.
108. **Cannon CG, and Linnemann Jr. CC.** 1992. *Yersinia enterocolitica* infections in hospitalized patients: the problem of hospital-acquired infections. *Infect Control Hosp Epidemiol* **13**: 139-143.
109. **Caprioli A, Pezzella C, Morelli R, Giammanco A, Arista S, Crotti D, Facchini M, Gugliemetti P, Piersimoni C, Luzzi I, and the Italian studygroup on gastrointestinal infections.** 1996. Enteropathogens associated with childhood diarrhoea in Italy. *Pediatr Infect Dis J* **15**: 876-883.
110. **Carlsson MG, Ryd H, and Sternby NH.** 1964. A case of human infection with *Pasteurella pseudotuberculosis X. Acta Pathol Microbiol Scand* **84**: 128-132.
111. **Carniel E.** 1995. Chromosomal virulence factors of *Yersinia*. *Contr Microbiol Immunol* **13**: 218-224.
112. **Carniel E, Guiyoule A, Guilvout I, and Mercereau-Puijalon O.** 1992. Molecular cloning, iron-regulation and mutagenesis of the *irp2* gene encoding HMWP2, a protein specific for the highly pathogenic *Yersinia*. *Mol Microbiol* **6**: 379-388.
113. **Carter PB.** 1981. Human *Yersinia enterocolitica* infection: Laboratory models. In: *Yersinia enterocolitica*. Bottone EJ (ed) CRC Press Inc, Boca Raton, Florida, USA, pp 73-81.
114. **Caugant DE, Aleksic S, Mollaret HH, Selander RK, and Kapperud G.** 1989. Clonal diversity and relationships among strains of *Yersinia enterocolitica*. *J Clin Microbiol* **27**: 2678-2683.
115. **CDC** (Centers for Disease Control). 1998a. *Yersinia enterocolitica*. In: US FDA/CFSAN Bad Bug Book. (http://vm.cfsan.fda.gov/~mow/chap5.html)
116. **CDC** (Centers for Disease Control). 1998b. Incidence of foodborne illnesses - FoodNet 1997. *MMWR* **47**: 782-786.
117. **CDC** (Centers for Disease Control). 1991. Update: *Yersinia enterocolitica* bacteraemia and endotoxin shock associated with red blood cell transfusions - United States 1991. *MMWR* **40**: 176-178.
118. **CDN** (Communicable Diseases Network) Australia New Zealand. National Notifiable Diseases Surveillance System. (http://www.health.gov.au/pubhlth/cdi/nndss/year042.htm)
119. **Challa VR, and Marx RS.** 1980. Pathology of *Yersinia enterocolitica* meningitis. *J Neurol Neurosurg Psychiatry* **43**: 455-457.
120. **Chambers CE, and Sokol PA.** 1994. Comparison of siderophore production and utilization in pathogenic and environmental isolates of *Yersinia enterocolitica*. *J Clin Microbiol* **32**: 32-39.

121. **Cheasty T, Said B, Jiggle B, and Threlfall J.** 1998. *Yersinia* species associated with diarrhoeal disease in the United Kingdom during the period 1990-1996. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S11.
122. **Chiesa C, Pacifico L, Cianfrano V, and Midulla M.** 1987. Italian experience with yersiniosis (1978-1985). *Contr Microbiol Immunol* **9**: 76-88.
123. **Chiesa C, Pacifico L, Guiyoule A, Ravagnan G, and Mollaret HH.** 1991. Phenotypic characterization and virulence of O:8 *Yersinia* strains isolated in Europe. *Contr Microbiol Immunol* **12**: 182-191.
124. **Christensen SG.** 1987a. Co-ordination of a nationwide survey on the presence of *Yersinia enterocolitica* O:3 in the environment of butcher shops. *Contr Microbiol Immunol* **9**: 26-29.
125. **Christensen SG.** 1987b. The *Yersinia enterocolitica* situation in Denmark. *Contr Microbiol Immunol* **9**: 93-97.
126. **Constantiniu S, Carniel E, Romaniuc, and Chiriac R.** 1998. Update of *Yersinia* infection in Romania. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S37-38.
127. **Daniëls JJHM, and Goudzwaard C.** 1963. Enkele stammen van een op *Pasteurella pseudotuberculosis* gelijkend niet geïdentificeerd species, geïsoleed bij knaagdieren. (In Dutch). <Some strains of a not identified species resembling *Pasteurella pseudotuberculosis*, isolated in rodents.> *Tijdschr Diergeneesk* **88**: 96-102.
128. **Davidson RJ, and Sprung DW.** 1989. Occurrence of *Listeria monocytogenes, Campylobacter* spp. and *Yersinia enterocolitica* in Manitoba raw milk. *Can Inst Food Sci Technol J* **22**: 70-74.
129. **Davies AR, and Slade A.** 1995. Fate of *Aeromonas* and *Yersinia* on modified-atmosphere-packaged (MAP) cod and trout. *Lett Appl Microbiol* **21**: 354-358.
130. **DeBuono BA.** 1995. Improper food preparation linked to infection in Buffalo children. New York State Department of Health, DOH-news. (http://www.health.state.ny.us/nysdoh/consumer/ pressrel/foodprep.htm)
131. **Delor I, and Cornelis GR.** 1992. Role of *Yersinia enterocolitica* Yst toxin in experimental infection in young rabbits. *Infect Immun* **60**: 4269-4277.
132. **Delor I, Kaeckenbeeck A, Wauters G, and Cornelis GR.** 1990. Nucleotide sequence of *yst*, the *Yersinia enterocolitica* gene encoding the heat-stable enterotoxin, and prevalence of the gene among pathogenic and non-pathogenic Yersiniae. *Infect Immun* **58**: 2983-2988.
133. **Desmasures N, Bazin F, and Guéguen M.** 1997. Microbiological composition of raw milk from selected farms in the Camembert region of Normandy. *J Appl Microbiol* **83**: 53-58.
134. **Dickinson AB, and Mocquot G.** 1961. Studies on the alimentary tract of pigs. *Enterobacteriaceae* and other Gram-negative bacteria. *J Appl Bacteriol* **24**: 252-284.
135. **Dmitrovsky AM, Dobritsa VP, Bezrukova LS, Necrasova LE, Meka-Mechenko TV, Kuchach IV, Dernovaya VF, Ryabushko EA, and Turegeldieva DA.** 1998. Epidemiological aspects of yersiniosis. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: 38.
136. **Doherty A, Sheridan JJ, Allen P, McDowell DA, Blair IS, and Harrington D.** 1995. Growth of *Yersinia enterocolitica* O:3 on modified atmosphere packaged lamb. *Food Microbiol* **12**: 251-257.
137. **Dolina M, and Peduzzi R.** 1993. Population genetics of human, animal and environmental *Yersinia* strains. *Appl Environ Microbiol* **59**: 442-450.
138. **Donald K, Woodson J, Hudson H, and Menzoian JO.** 1996. Multiple mycotic pseudoaneurysms due to *Yersinia enterocolitica*: report of a case and review of the literature. *Ann Vas Surg* **10**: 573-577.
139. **Doyle MP, Hugdahl MB, and Taylor SL.** 1981. Isolation of virulent *Yersinia enterocolitica* from porcine tongues. *Appl Environ Microbiol* **42**: 661-666.
140. **Dziezak JD.** 1991. USDA develops tests for quick detection of *Yersinia*. *Food Technol* **45**: 95-96.
141. **Durisin MD, Ibrahim A, and Griffiths MW.** 1997. Detection of pathogenic *Yersinia enterocolitica* in milk and pork using a DIG-labelled probe targeted against the *yst* gene. *Int J Food Microbiol* **37**: 103-112.

142. **Eden KV, Rosenberg ML, Stoopler M, Wood BT, Highsmith AK, Skaliy P, Wells JG, and Feeley JC.** 1977. Waterborne gastrointestinal illness at a ski resort: isolation of *Yersinia enterocolitica* from drinking water. *Public Health Rep* **92**: 245-250.
143. **Egorov I, Maramovich AS, Cherniavskii VF, Chesnokova MV, and Tupitsina AF.** 1996. Epidemiologicheskie zakonomernosti iersiniozov v Respublike Sakha (Iakutiia). (In Russian). <The epidemiological patterns of yersiniosis in the Republic of Sakha (Yakutia).> *Zhurn Mikrobiol Epidemiol Immunol* **2**: 40-43.
144. **Eiss J.** 1975. Selective culture of *Yersinia enterocolitica* at low temperature. *Scand J Inf Dis* **7**: 249-251.
145. **Elrick J.** 1988. A case-study: fatal Gram-negative shock following blood transfusion. *Yersinia News*, Melbourne, Australia, **5**: 1-2.
146. **Erickson JP.** 1995. Behaviour of *Yersinia enterocolitica* in commercially pasteurized liquid eggs held at 2, 6.7 and 12.8°C. *Contr Microbiol Immunol* **13**: 83-85.
147. **Escudero ME, Velázquez LC del, and de Guzmán AMS.** 1996. *Yersinia enterocolitica* and related species isolated from animals slaughtered for human consumption. *Food Microbiol* **13**: 201-204.
148. **Esseveld H, and Goudzwaard C.** 1973. On the epidemiology of *Yersinia enterocolitica* infections: pigs as the source of infections in man. *Contr Microbiol Immunol* **2**: 99-101.
149. **Esveld MI, van Pelt W, van Leeuwen WJ, and Bänffer JRJ.** 1996. Laboratorium Surveillance Infectieziekten 1989-1995. (In Dutch) <Laboratory surveillance infectious diseases.> National Institute of Public Health and the Environment, Bilthoven, the Netherlands. *RIVM report* nr. 968902002.
150. **Falcão DP, Machado de Medeiros BM, de Moraes Costa A, and Araújo PMF.** 1995. Presence of autoantibodies in the serum of mice infected with *Yersinia enterocolitica*. *Contr Microbiol Immunol* **13**: 195-198.
151. **Fantasia M, Mingrone MG, Crotti D, and Boscato C.** 1985. Isolation of *Yersinia enterocolitica* biotype 4 serotype O:3 from canine sources in Italy. *J Clin Microbiol* **22**: 314-315.
152. **Fantasia M, Mingrone MG, Martini A, Boscato U, and Crotti D.** 1993. Characterization of *Yersinia* species isolated from a kennel and from cattle and pig farms. *Vet Rec* **132**: 532-534.
153. **Farmer III JJ, Carter GP, Miller VL, Falkow S, and Wachsmuth IK.** 1992. Pyrazinamidase, CR-MOX agar, salicin fermentation-esculin hydrolysis, and D-xylose fermentation for identifying pathogenic serotypes of *Yersinia enterocolitica*. *J Clin Microbiol* **30**: 2589-2594.
154. **Fenwick SG, Madie P, and Wilks CR.** 1994. Duration of carriage and transmission of *Yersinia enterocolitica* biotype 4, serotype O:3 in dogs. *Epidemiol Infect* **113**: 471-477.
155. **Fenwick SG.** 1998. *Yersinia enterocolitica* infections in animals and people in New Zealand. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S12.
156. **Fenwick SG, and McCarthy MD.** 1995. *Yersinia enterocolitica* is a common cause of gastroenteritis in Auckland. *NZ Med J* **108**: 269-271.
157. **Fenwick SG, Wauters G, Ursing J, and Gwozdz M.** 1996. Unusual *Yersinia enterocolitica* strains recovered from domestic animals and people in New Zealand. *FEMS Immunol Med Microbiol* **16**: 241-245.
158. **Figura N, and Rossolini A.** 1985. A prospective etiological and clinical study on gastroenteritis in Italian children. *Boll Ist Sieroter Milan* **64**: 302-310.
159. **Figura N, Guglielmetti P, Zanchi A, Signori R, Rossolini A, Lior H, Russi M, and Musmanno RA.** 1997. Species, biotype and serogroup of *Campylobacter* spp. isolated from children with diarrhoea over a ten-year period. *Microbiologica (New Microbiol)* **20**: 303-310.
160. **Finlay BB, and Cossart P.** 1997. Exploitation of mammalian host cell functions by bacterial pathogens. *Science* **276**: 718-725.
161. **Fliss I, Blais BW, Holley R, and Simard RE.** 1995. Multiplex riboprobes for the detection of virulent *Yersinia enterocolitica* and simple methods for their preparation. *J Appl Bacteriol* **79**: 195-202.
162. **Francis DW, Spaulding PL, and Lovett J.** 1980. Enterotoxin production and thermal resistance of *Yersinia enterocolitica* in milk. *Appl Environm Microbiol* **40**: 174-176.
163. **Fransen NG, van den Elzen AMG, Urlings BAP, and Bijker PGH.** 1996. Pathogenic micro-

organisms in slaughterhouse sludge - a survey. *Int J Food Microbiol* **33**: 245-256.
164. **Franzin L, and Curti F.** 1993. Prevalence of *Yersinia* antibodies in blood donors. *Eur J Epidemiol* **9**: 224-228.
165. **Franzin L, Di-Rosa G, and Sinicco A.** 1987. Attempt of *Yersinia enterocolitica* isolation from human faeces in Senegal. *Eur J Epidemiol* **3**: 445-447.
166. **Franzin L, Fora R, and Sinicco A.** 1998. *Yersinia* antibodies and *Yersinia* carriage in slaughterhouse workers. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S49.
167. **Franzin L, and Gioannini P.** 1992. Growth of *Yersinia* species in artificially contaminated blood bags. *Transfusion* **32**: 673-676.
168. **Frederiksen W.** 1964. A study of some *Yersinia pseudotuberculosis*-like bacteria ('*Bacterium enterocoliticum*' and '*Pasteurella X*'). *Proc 14th Scand Congr Pathol Microbiol*, Oslo, Norway. pp 103-104.
169. **Fredriksson-Ahomaa M, Björkroth J, and Korkeala H.** 1998. Characterization of *Yersinia enterocolitica* bioserotype 4/O:3 from pig tongues by PFGE. Abstracts of the 7th Int congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S14.
170. **Fukushima H, and Gomyoda M.** 1986. Inhibition of *Yersinia enterocolitica* serotype O:3 by natural microflora of pork. *Appl Environm Microbiol* **51**: 990-994.
171. **Fukushima H, Hoshina K, Nakamura R, Ito Y, and Gomyoda M.** 1987. Epidemiological study of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in Shimane prefecture, Japan. *Contr Microbiol Immunol* **9**: 103-110.
172. **Fukushima H, Hoshina K, Itogawa H, and Gomyoda M.** 1997. Introduction into Japan of pathogenic *Yersinia* through imported pork, beef and fowl. *Int J Food Microbiol* **35**: 205-212.
173. **Fukushima H, Saito K, Tsubokura M, and Otsuki K.** 1984. *Yersinia* spp. in surface water in Matsue, Japan. *Zentralbl Bakteriol Mikrobiol Hyg B* **179**: 236-247.
174. **Funk JA, Troutt HF, Isaacson RE, and Fossler CP.** 1998. Prevalence of pathogenic *Yersinia enterocolitica* in groups of swine at slaughter. *J Food Prot* **61**: 677-682.
175. **García de Fernando GD, Nychas GJE, Peck MW, and Ordóñez JA.** 1995. Growth/survival of psychrotrophic pathogens on meat packaged under modified atmospheres. *Int J Food Microbiol* **28**: 221-231.
176. **Garcia-Jalon I, Muro L, Leiva J, and Diaz R.** 1998. (In Spanish). <Study of *Yersinia enterocolitica* in pork.> *Alimentaria* **292**: 27-31.
177. **Gauthier T, and So AK.** 1997. Vasculitis and bacteraemia with *Yersinia enterocolitica* in late-onset systemic lupus erythematosus. *Br J Rheum* **36**: 1122-1124.
178. **Gemski P, Lazere JR, and Casey T.** 1980. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. *Infect Immun* **27**: 682-685.
179. **Giamarellou H, Antoniadou A, Kanavos K, Papaioannou C, Kanatakis S, and Papadaki K.** 1995. *Yersinia enterocolitica* endocarditis: case report and literature review. *Eur J Clin Microbiol Infect Dis* **14**: 126-130.
180. **Gilbert R.** 1940. Diagnostic laboratoires: an unidentified microorganism resembling *Actinobacillus ligneresi* and *Pasteurella pseudotuberculosis*, and pathogenic for man. *NY State Dep Health, Ann Rep Divis Labor Res* **XX**: 50-51.
181. **Glenn Morris Jr. JG, Prado V, Ferreccio C, Robins-Browne RM, Bordun AM, Cayazzo M, Kay BA, and Levine MM.** 1991. *Yersinia enterocolitica* isolated from two cohorts of young children in Santiago, chile: incidence and lack of correlation between illness and proposed virulence factors. *J Clin Microbiol* **29**: 2784-2788.
182. **González Hevia MA, Alvarez Riesgo JA, and Mendoza MC.** 1990. Epidemiological, clinical and microbiological features of *Yersinia enterocolitica* infections in a community during a four-year period. *Eur J Epidemiol* **6**: 184-190.
183. **Granfors K, Jalkanen S, von Essen R, Lahesmaa-Rantala R, Isomäki O, Pekkola-Heino K, and Toivanen A.** 1989. *Yersinia* antigens in synovial-fluid cells from patients with reactive arthritis. *N Eng J Med* **320**: 216-221.
184. **Gray LD.** 1995. *Escherichia, Salmonella, Shigella,* and *Yersinia*. In: *Manual of clinical*

Microbiology, 6th ed. Murray PR (ed), p 455.
185. **Greenwood MH.** 1995. Human carriage of *Yersinia* species and incidence in foods. *Contr Microbiol Immunol* **13**: 74-76.
186. **Greenwood MH, and Hooper WL.** 1987. Human carriage of *Yersinia* spp. *J Med Microbiol* **23**: 345-348.
187. **Greenwood MH, and Hooper WL.** 1990. Excretion of *Yersinia* spp. associated with consumption of pasteurized milk. *Epidemiol Infect* **104**: 345-350.
188. **Greer GG, Gill CO, and Dilts BD.** 1995. Predicting the aerobic growth of *Yersinia enterocolitica* on pork fat and muscle tissues. *Food Microbiol* **12**: 463-469.
189. **Guilvout I, Mercereau-Puijalon O, Bonnefoy S, Pugsley AP, and Carniel E.** 1993. High-molecular-weight protein 2 of *Yersinia enterocolitica* is homologous to AngR of *Vibrio anguillarum* and belongs to a family of proteins involved in nonribosomal peptide synthesis. *J Bacteriol* **175**: 5488-5504.
190. **Gurgui M, Mirelis B, Coll P, and Prats G.** 1988. Epidemiology of Yersiniae in Barcelona, Spain. *Microbiologia* **4**: 107-116.
191. **Gutman LT, Ottesen EA, Quan TJ, Noce PS, and Katz SL.** 1973. An inter-familial outbreak of *Yersinia enterocolitica* enteritis. *New Eng J Med* **288**: 1372-1377.
192. **Haghighi L.** 1979. The first successful isolation and identification of *Yersinia enterocolitica* in Iran. *Contr Microbiol Immunol* **5**: 206-211.
193. **Halpin TJ, Moore W, Waterman SH, Hadler JL, Wilcox KR, *et al.*.** 1997. Red blood cell transfusions contaminated with *Yersinia enterocolitica* - United States 1991-1996, and initiation of a national study to detect bacteria-associated transfusion reactions. *JAMA* **278**: 196-197.
194. **Hanna MO, Stewart JC, Zink DL, Carpenter ZL, and Vanderzant C.** 1977. Development of *Yersinia enterocolitica* on raw and cooked beef and pork at different temperatures. *J Food Sci* **42**: 1180-1184.
195. **Hanna MO, Stewart JC, Carpenter ZL, and Vanderzant C.** 1979. Effect of heating, freezing and pH on *Yersinia enterocolitica*-like organisms from meat. *J Food Prot* **40**: 689-692.
196. **Hanski C, Kutschka V, Schmoranzer HP, Naumann M, Stallmach A, Hahn H, Menge H, and Riecken EO.** 1987. Immunohistochemical and electron microscopic study of interaction of *Yersinia enterocolitica* serotype O:8 with intestinal mucosa during experimental enteritis. *Infect Immun* **57**: 673-678.
197. **Harnett N, Lin YP, and Krishnan C.** 1996. Detection of pathogenic *Yersinia enterocolitica* using the multiplex polymerase chain reaction. *Epidemiol Infect* **117**: 59-67.
198. **Harnett N, Hu G, Wan J, Brunins V, Borczyk A, and Jamieson F.** 1998. Epidemiology of *Yersinia enterocolitica* infections in Canada. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S12.
199. **Hässig AJ von, Karrer J and Pusterla F.** 1949. Über Pseudotuberkulose beim Menschen. *Schweiz Med Wochenschr* **79**: 971-973.
200. **Haverly RM, Harrison CR, and Dougherty TH.** 1996. *Yersinia enterocolitica* bacteraemia associated with red blood cell transfusion. *Arch Pathol Lab Med* **120**: 499-500.
201. **Hayashidani H, Ohtomo Y, Toyokawa Y, Saito M, Kaneko KI, Kosuge J, Kato M, Ogawa M, and Kapperud G.** 1995. Potential sources of sporadic human infection with *Yersinia enterocolitica* serovar O:8 in Aomori prefecture, Japan. *J Clin Microbiol* **33**: 1253-1257; *Contr Microbiol Immunol* **13**: 29-32.
202. **Head CB, Whitty DA, and Ratnam S.** 1982. Comparative study of selective media for recovery of *Yersinia enterocolitica*. *J Clin Microbiol* **16**: 615-621.
203. **Heesemann J, Keller C, Morawa R, Schmidt N, Siemens HJ, and Laufs R.** 1983. Plasmids of human strains of *Yersinia enterocolitica*: molecular relatedness and possible importance for pathogenesis. *J Infect Dis* **147**: 107-115.
204. **Heesemann J.** 1990. Enteropathogene Yersinien: Pathogenitätsfaktoren und neue diagnostische Methoden. *Immun Infekt* **18**: 186-191.
205. **Heesemann J, Schubert S, Roggenkamp A, Rakin A, and Autenrieth I.** 1997. Pathogenicity of *Yersinia*: a strategy for extracellular survival and multiplication. In: *Ecology of pathogenic*

bacteria; molecular and evolutionary aspects. Van der Zeijst BAM, Hoekstra WPM, van Embden JDA, and van Alphen AJW (eds). KNAW Lectures, Dep Physica, 2nd Ser, Amsterdam, The Netherlands. Vol 96: 129-141.

206. **Herbes RG, Edel W, Komijn RE, and Smak JA.** 1995. *Zoönosen en zoönosenverwekkers in Nederland*. Ministerie van Volksgezondheid, Welzijn en Sport; Ministerie van Landbouw Natuurbeheer en Visserij. pp 1-33.
207. **Herbes RG, Nieuwenhuijs JHM, and Komijn RE.** 1999. *Zoönosen en zoönosenverwekkers in Nederland*. Ministerie van Volksgezondheid, Welzijn en Sport; Ministerie van Landbouw Natuurbeheer en Visserij. p 54.
208. **Heyden IM van der, Res PCM, Wilbrink B, Leow A, Breedveld FC, Heesemann J, and Tak PP.** 1997. *Yersinia enterocolitica*: a cause of chronic polyarthritis. *Clin Infect Dis* **25**: 831-837.
209. **Hill WE, Payne WL, and Aulisio CCG.** 1983. Detection and enumeration of virulent *Yersinia enterocolitica* in food by DNA colony hybridization. *Appl Environ Microbiol* **46**: 636-641.
210. **Hoogkamp-Korstanje JAA, de Koning J, and Samsom JP.** 1986. Incidence of human infection with *Yersinia enterocolitica* serotypes O:3, O:8 and O:9 and the use of indirect immunofluorescence in diagnosis. *J Infect Dis* **153**: 138-141.
211. **Hosaka S, Uchiyama M, Ishikawa M, Akahoshi T, Kondo H, Shimauchi C, Sasahara T, and Inoue M.** 1997. *Yersinia enterocolitica* serotype O:8 septicemia in an otherwise healthy adult. *J Clin Microbiol* **35**: 3346-3347.
212. **Househam KC, Mann MD, and Bowie MD.** 1988. Enteropathogens associated with acute infantile diarrhoea in Cape Town. *S Afr Med J* **73**: 83-87.
213. **Huang X, Yoshino K, Nakao H, and Takeda T.** 1997. Nucleotide sequence of a gene encoding the novel *Yersinia enterocolitica* heat-stable enterotoxin that includes a pro-region-like sequence in its mature toxin molecule. *Microbial Pathogenesis* **22**: 89-97.
214. **Hudson JA, and Avery SM.** 1994. Growth of *Listeria monocytogenes*, *Aeromonas hydrophila* and *Yersinia enterocolitica* on cooked mussel tissue under refrigeration and mild temperature abuse. *J Food Safety* **14**: 41-52.
215. **Hudson JA, and Mott SJ.** 1993. Growth of the cold-tolerant bacteria *Listeria monocytogenes*, *Aeromonas hydrophila* and *Yersinia enterocolitica* on cold-smoked salmon under refrigeration and mild temperature abuse. *Food Microbiol* **10**: 61-68.
216. **Hurvell B.** 1981. Zoonotic *Yersinia enterocolitica* infection: host range, clinical manifestations, and transmission between animals and man. In: Bottone EJ (ed) *Yersinia enterocolitica*. CRC Press Inc, Boca Raton, Florida. pp 145-159.
217. **Ibrahim A, Liesack W, and Stackebrandt E.** 1992a. Differentiation between pathogenic and non-pathogenic *Yersinia enterocolitica* strains by colony hybridization with a PCR-mediated digoxigenin-dUTP-labelled probe. *Mol Cell Probes* **6**: 163-171.
218. **Ibrahim A, Liesack W, and Stackebrandt E.** 1992b. Polymerase chain reaction-gene probe detection system specific for pathogenic strains of *Yersinia enterocolitica*. *J Clin Microbiol* **30**: 1942-1947.
219. **Ibrahim A, Liesack W, Griffiths MW, and Robins-Browne RM.** 1997. Development of a highly specific assay for rapid identification of pathogenic strains of *Yersinia enterocolitica* based on PCR amplification of the *Yersinia* heat-stable enterotoxin gene (*yst*). *J Clin Microbiol* **35**: 1636-1638.
220. **Ichinohe H, Yoshioka M, Fukushima H, Kaneko S, and Maruyama T.** 1991. First isolation of *Yersinia enterocolitica* serotype O:8 in Japan. *J Clin Microbiol* **29**: 846-847.
221. **Iriarte M, and Cornelis GR.** 1998. YopT, a new *Yersinia* effector protein, affects the cytoskeleton of host cells. *Mol Microbiol* **29**: 915-929.
222. **Iriarte M, Stainier I, and Cornelis GR.** 1995. The *rpoS* gene from *Yersinia enterocolitica* and its influence on expression of virulence factors. *Infect Immun* **63**: 1840-1847.
223. **Iriarte M, Vanooteghem JC, Delor I, Díaz R, Knutton S, and Cornelis GR.** 1993. The Myf fibrillae of *Yersinia enterocolitica*. *Mol Microbiol* **9**: 507-520.
224. **Iteman I, Guiyoule A, and Carniel E.** 1996. Comparison of three molecular methods for typing and subtyping pathogenic *Yersinia enterocolitica* strains. *J Med Microbiol* **45**: 48-56.

225. **Jacobs J, Jamaer D, Vandeven J, Wouters M, Vermylen C, and Vandepitte J.** 1989. *Yersinia enterocolitica* in donor blood: a case report and review. *J Clin Microbiol* **27**: 1119-1121.
226. **Jagow J, and Hill WE.** 1986. Enumeration by DNA hybridization of virulent *Yersinia enterocolitica* colonies in artificially contaminated food. *Appl Environ Microbiol* **51**: 441-443.
227. **Jegathesan M, Paramasivam T, Rajagopalan K, and Lim KL.** 1984. *Yersinia enterocolitica* infection: first case report from Malaysia. *Trop Geogr Med* **36**: 207-210.
228. **Jennings DL, Miliotis MD, and Koornhof HJ.** 1987.Human *Yersinia enterocolitica* infection in eastern Cape Town. A report on 18 cases and a review of yersiniosis in the RSA. *S Afr Med J* **72**: 868-870.
229. **Jensen KT, Arpi M, and Frederiksen W.** 1995. *Yersinia enterocolitica* septicemia in Denmark 1972-1991: a report of 100 cases. *Contr Microbiol Immunol* **13**: 11-15.
230. **Johannessen G, Kapperud G, and Kruse H.** 1998. The occurrence of pathogenic *Yersinia enterocolitica* in Norwegian pork products measured by the use of a traditional culturing method and PCR. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S14.
231. **Kallinowski F, Wassmer A, Hofmann M, Harmsen D, Heesemann J, Karch H, Herfarth C, and Buhr H.** 1998. Prevalence of enteropathogenic bacteria in surgically treated inflammatory bowel disease. *Hep Gastroenterol* **45**: 1552-1558.
232. **Kandolo K, and Wauters G.** 1985. Pyrazinamidase activity in *Yersinia enterocolitica* and related organisms. *J Clin Microbiol* **21**: 980-982.
233. **Kanazawa Y, and Ikemura K.** 1979. Isolation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from human specimens and their drug-resistance in the Niagata district of Japan. *Contr Microbiol Immunol* **5**: 106-114.
234. **Kaneko S, Ishizaki N, Higashide M, and Namekata C.** 1998. Changes in biotypes of *Yersinia enterocolitica* serotype O:3 isolated from humans in Japan. Abstracts of the 7th Int congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S45.
235. **Kapperud G, Dommarsnes K, Skurnik M, and Hornes E.** 1990. A synthetic oligonucleotide probe and a cloned polynucleotide probe based on the *YopA* gene for detection and enumeration of virulent *Yersinia enterocolitica*. *Appl Environ Microbiol* **56**: 17-23.
236. **Kapperud G, and Langeland G.** 1981. Enterotoxin production at refrigeration temperature by *Yersinia enterocolitica* and *Yersinia enterocolitica*-like bacteria. *Curr Microbiol* **5**: 119-122.
237. **Kapperud G, Nesbakken T, Aleksić S, and Mollaret HH.** 1990. Comparison of restriction endonuclease analysis and phenotypic typing methods for differentiation of *Yersinia enterocolitica* isolates. *J Clin Microbiol* **28**: 1125-1131.
238. **Kapperud G, Ostroff SM, Nesbakken T, Hutwagner LC, Bean NH, Lassen T, and Tauxe RV.** 1995. Risk factors for sporadic *Yersinia enterocolitica* infections in Norway: a case-control study. *Contr Microbiol Immunol* **13**: 25-28.
239. **Karapinar M, and Gönül SA.** 1991. Survival of *Yersinia enterocolitica* and *Escherichia coli* in spring water. *Int J Food Microbiol* **13**: 315-320.
240. **Kasatiya SS.** 1976. *Yersinia enterocolitica* gastroenteritis outbreak - Montreal. *Can Dis Wkly Rep* 2: 73-74,
241. **Keet EE.** 1974. *Yersinia enterocolitica* septicemia. Source of infection and incubation period identified. *NY State J Med* **74**: 2226-2230.
242. **Kendall M, and Gilbert RJ.** 1980. Survival and growth of *Yersinia enterocolitica* in broth media and in food. In: *Microbial growth and survival in extremes of environment.* Gould GW, and Corry JEL (eds). Soc Appl Bacteriol Techn Ser, Academic Press, London, UK. Vol 15: pp 217-226.
243. **Kleemann J, and Bergann T.** 1996. Model experiments to establish behaviour of *Yersinia enterocolitica* O:9 strains in various types of fresh dry sausage. *J Appl Bacteriol* **80**: 10-12.
244. **Kleinlein N, and Untermann F.** 1990. Growth of pathogenic *Yersinia enterocolitica* strains in minced meat with and without protective gas with consideration of the competitive background flora. *Int J Food Microbiol* **10**: 65-72.
245. **Knapp W, and Thal E.** 1963. Untersuchungen über die kulturell-biochemischen, serologischen, tierexperimentellen und immunologischen Eigenschaften einer vorläufig '*Pasteurella X*' benannten Bakterienart. *Zbl Bakteriol Parasitenkd Infektionskr Hyg Abt 1 Orig* **190**: 472-484.

246. Knapp W, and Thal E. 1973. Differentiation of *Yersinia enterocolitica* by biochemical reactions. *Contr Microbiol Immunol* 2: 10-16.
247. Kontiainen S, Sivonen A, and Renkonen OV. 1994. Increased yields of pathogenic *Yersinia enterocolitica* strains by cold enrichment. *Scand J Infect Dis* **26**: 685-691.
248. Kotloff KL, Wasserman SS, Steciak JY, Tall BD, Losonsky GA, Nair P, Glenn Morris Jr. J, and Levine MM. 1988. Acute diarrhoea in Baltimore children attending an outpatient clinic. *Pediatr Infect Dis J* **7**: 753-759.
249. Kotula AW, and Sharar AK. 1993. Presence of *Yersinia enterocolitica* serotype O:5,27 in slaughter pigs. *J Food Prot* **56**: 215-218.
250. Kristensen M, Bojlén K, and Kjæer T. 1935. Systematische Untersuchungen über coliähnliche Bakterien. *Zbl Bakteriol I Abt Orig* **134**: 318-340.
251. Kuehnert MJ, Jarvis WR, Schaffer DA, and Chaffin DJ. 1997. Platelet transfusion reaction due to *Yersinia enterocolitica*. *JAMA* **278**: 550.
252. Kwaga J, Iversen JO, and Misra V. 1992. Detection of pathogenic *Yersinia enterocolitica* by polymerase chain reaction and digoxigenin-labelled polynucleotide probes. *J Clin Microbiol* **30**: 2668-2673.
253. La Scola B, Musso D, Carta A, Piquet P, and Casalta JP. 1997. Aortoabdominal aneurysm infected by *Yersinia enterocolitica* serotype O:9. *J Infect* **35**: 314-315.
254. Lanciotti R, Massa S, Guerzoni ME, and DiFabio G. 1992. Light butter: natural microbial population and potential growth of *Listeria monocytogenes* and *Yersinia enterocolitica*. *Lett Appl Microbiol* **15**: 256-258.
255. Laird WJ, and Cavanaugh DC. 1980. Correlation of autoagglutination and virulence of *Yersinia enterocolitica*. *J Clin Microbiol* **11**: 430-432.
256. Laiskonis A, Juceviciene A, Velyvyte D, and Krisciuniene I. 1998. *Yersinia enterocolitica* infection in Kaunas (Lithuania). Abstracts of the 7th Int congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S37.
257. Larsen JH. 1979. The spectrum of clinical manifestations of infections with *Yersinia enterocolitica* and their pathogenesis. *Contr Microbiol Immunol* **5**: 257-269.
258. Larsen JH. 1980. *Yersinia enterocolitica* infections and rheumatic diseases. *Scan J Rheumatology* **9**: 129-137.
259. Larsen JH, Hartzen SH, and Parm M. 1985. The determination of specific IgA-antibodies to *Yersinia enterocolitica* and their role in enteric infections and their complications. *Acta Path Microbiol Immunol Scand Sect B* **93**: 331-339.
260. Larkin LL, Vasavada PC, and Marth EH. 1991. Incidence of *Yersinia enterocolitica* in raw milk as related to its quality. *Milchwissenschaft* **46**: 500-502.
261. Lassen J. 1972. *Yersinia enterocolitica* in drinking water. *Scand J Infect Dis* **4**: 125-127.
262. Lee LA, Gerber AR, Lonsway DR, Smith JD, Carter GP, Puhr ND, Parrish CM, Sikes RK, Finton RJ, and Tauxe RV. 1990. *Yersinia enterocolitica* O:3 infections in infants and children, associated with the household preparation of chitterlings. *N Engl J Med* **322**: 984-987.
263. Lee LA, Taylor J, Carter GP, Quinn B, Farmer III JJ, and Tauxe RV. 1991. *Yersinia enterocolitica* O:3: an emerging cause of pediatric gastroenteritis in the United States. *J Inf Dis* **163**: 660-663.
264. Leistner L, Hechelmann H, Kashiwazaki M, and Albertz R. 1975. Nachweis von *Yersinia enterocolitica* in Faeces und Fleisch von Schweinen, Rinder und Geflügel. *Fleischwirts* **11**: 1599-1602.
265. Lewis AM, and Chattopadhyay B. 1986. Faecal carriage rate of *Yersinia* species. *J Hyg Camb* **97**: 281-287.
266. Little CL, Adams MR, and Easter MC. 1992. The effect of pH, acidulant and temperature on the survival of *Yersinia enterocolitica*. *Lett Appl Microbiol* **14**: 148-152.
267. Little CL, and Knochel S. 1994. Growth and survival of *Yersinia enterocolitica*, *Salmonella* and *Bacillus cereus* in brie stored at 4, 8 and 20 degrees. *Int J Food Microbiol* **24**: 137-145.
268. Lobato MJ, Landeras E, González Hevia MA, and Mendoza MC. 1998. Genetic heterogeneity of clinical strains of *Yersinia enterocolitica* traced by ribotyping and relationships between ribotypes, serotypes and biotypes. *J Clin Microbiol* **36**: 3297-3302

269. **Logue CM, Sheridan JJ, Wauters G, McDowell DA, and Blair IS.** 1996. *Yersinia* spp. and numbers, with particular reference to *Y. enterocolitica* bio/serotypes, occurring in Irish meat and meat products, and influence of alkali treatment on their isolation. *Int J Food Microbiol* **33**: 257-274.
270. **Mäki-Ikola O, Heesemann J, Toivanen A, and Granfors K.** 1997. High frequency of *Yersinia* antibodies in healthy populations in Finland and Germany. *Rheumatol Int* **16**: 227-229.
271. **Makulu A, Gatti F, Mollaret HH, and Vandepitte J.** 1969. Sur l'existence d'infection humaines a *Yersinia enterocolitica* en République Démocratique du Congo. *Bull Soc Pathol Exot* **62**: 452-460.
272. **Märker-Hermann E, and Höhler T.** 1998. Pathogenesis of human leucocyte antigen B27-positive arthritis. *Rheum Dis Clin North Am* **24**: 865-881.
273. **Markov IS, Tkachenko VI, and Silin DD.** 1989. Vspyshki psevdotuberkuleza i kishechnogo iersinioza sredi sovetskikh spetsialistov ichlenov ikh semei v Mongol'skoi Narodnoi Respublike (In Russian). <Outbreaks of pseudotuberculosis and intestinal yersiniosis among Soviet specialists and members of their families in the Mongolian People's republic.> *Zhurnal Mikrobiologii, Epidemiologii i Immunologii* **8**: 43-49.
274. **Marks MI, Pai CH, Lafleur L, Lackman L, and Hammerberg O.** 1980. *Yersinia enterocolitica* gastroenteritis. A prospective study of clinical, bacteriologic and epidemiologic features. *J Pediatr* **96**: 26-31.
275. **Marriot D.** 1987. *Yersinia enterocolitica* infection in children in New South Wales. *Contr Microbiol Immunol* **9**: 98-102.
276. **Martin T, Kasian GF, and Stead S.** 1982. Family outbreak of yersiniosis. *J Clin Microbiol* **16**: 622-626.
277. **Maruyama T.** 1987. *Yersinia enterocolitica* infection in humans and isolation of the microorganism from pigs in Japan. *Contr Microbiol Immunol* **9**: 48-55.
278. **Massa S, Cesaroni D, Poda G, and Trovatelli LD.** 1988. Isolation of *Yersinia enterocolitica* and related species from river water. *Zentralbl Mikrobiol* **143**: 575-581.
279. **McCarthy MD, and Fenwick SG.** 1990. Experiences with the diagnosis of *Yersinia enterocolitica* - an emerging gastrointestinal pathogen in the Auckland area 1987-1989. *NZ J Med Lab Sci* **45**: 19-22.
280. **McDonald CP, Barbara JA, Hewitt PE, Hartley S, Telfer P, Gale R, James E, and Prentice HG.** 1996. *Yersinia enterocolitica* transmission from a red cell unit 34 days old. *Transfus Med* **6**: 61-63.
281. **McIntyre M, and Nnochiri E.** 1986. A case of hospital-acquired *Yersinia enterocolitica* gastroenteritis. *J Hosp Infect* **7**: 299-301.
282. **McIver MA, and Pike RM.** 1934. Chronic glanders-like infection of face caused by an organism resembling *Flavobacterium pseudomallei* Whitmore. In: *Clinical Miscellany*, Vol 1. Mary Imogene Basset Hospital, Cooperstown N.Y., USA, pp 16-21.
283. **Meka-Mechenko TV, Necrasova LE, Dernovaya VF, Atshabar BB, and Arykbaeva UT.** 1998. Frequency of isolating *Yersiniae* from various human substrates. Abstracts of the 7th Int congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S44-45.
284. **Mendoza MC, Alzugaray R, Landeras E, and González-Hevia MA.** 1996. Discriminatory power and application of ribotyping of *Yersinia enterocolitica* O:3 in an epidemiological study. *Eur J Clin Microbiol Infect Dis* **15**: 220-226.
285. **Mercié P, Morlat P, N'Gako A, Phéline P, Bézian MC, Gorin G, *et al.*** 1996. Anévrismes aortiques à *Yersinia enterocolitica*:: trois nouveaux cas et revue de la littérature. *J Mal Vasc* **21**: 68-71.
286. **Merilahti-Palo R, Lahesmaa R, Granfors K, Gripenberg-Lerche C, and Toivanen P.** 1991. Risk of *Yersinia* infection among butchers. *Scand J Inf Dis* **23**: 55-61.
287. **Mertz AKH, Daser A, Skurnik M *et al.*** 1994. The evolutionarily conserved ribosomal protein L23 and the cationic urease ß-subunit of *Yersinia enterocolitica* O:3 belong to the immunodominant antigens in *Yersinia*-triggered reactive arthritis: Implications for autoimmunity. *Mol Med* **1**: 44-55.
288. **Mewis A, Verhaegen J, Bourgeois S, Hiele M, and Schelstraete B.** 1997. Fatal *Yersinia enterocolitica* transfusion reaction. *J Clin Pathol* **50**: 796.

289. **Mikulskis AV, Delor I, Thi VH, and Cornelis GR.** 1994. Regulation of the *Yersinia enterocolitica* enterotoxin Yst gene. Influence of growth phase, temperature, osmolarity, pH and bacterial host factors. *Mol Microbiol* **14**: 905-915.
290. **Miliotis MD, Galen JE, Kaper JB, and Glenn Morris Jr. JG.** 1989. Development and testing of a synthetic oligonucleotide probe for the detection of pathogenic *Yersinia* strains. *J Clin Microbiol* **27**: 1667-1670.
291. **Miller AJ, Call JE, and Eblen BS.** 1997. Growth, injury and survival potential of *Yersinia enterocolitica*, *Listeria monocytogenes* and *Staphylococcus aureus* in brine chiller conditions. *J Food Prot* **60**: 1334-1340.
292. **Miller VL, and Falkow S.** 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. *Infect Immun* **56**: 1242-1248.
293. **Miller VL, Farmer III JJ, Hill WE, Falkow S.** 1989. The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. *Infect Immun* **57**: 121-131.
294. **Mills SD, Boland A, Sory MP, Smissen P van der, Kerbourch C, Finlay BB, and Cornelis GR.** 1997. *Yersinia enterocolitica* induces apoptosis in macrophages by a process requiring functional type III secretion and translocation mechanisms and involving YopP, presumably acting as an effector protein. *Proc Natl Acad Sci USA* **94**: 12638-12643.
295. **Mingrone MG, Fantasia M, Figura N, and Guglielmetti P.** 1987. Characteristics of *Yersinia enterocolitica* isolated from children with diarrhoea in Italy. *J Clin Microbiol* **25**: 1301-1304.
296. **Mollaret HH, Chevalier A, and Deplanche MC.** 1964. Contribution à l'étude d'un nouveau groupe de germes proches du bacille de Malassez et Vignal. *Ann Inst Pasteur* **107**: 121-127.
297. **Mollaret HH, and Destombes P.** 1964. Les germes X en pathologie humaine. *Presse Méd* **72**: 2913-2915.
298. **Mollaret HH.** 1966. L'infection humaine à *Yersinia enterocolitica*. *Path Biol* **14**: 981-990.
299. **Mollaret HH.** 1976. Contribution à l'étude épidémiologique des infections à *Yersinia enterocolitica*. III. Bilan provisoire des connaissances. *Méd Mal Infect* **6**: 442-448.
300. **Mollaret HH.** 1983. *Yersinia pseudotuberculosis* et *Yersinia enterocolitica*. *Rev Rhum* **50**: 713-717.
301. **Mollaret HH, Alonso JM, and Bercovier H.** 1976. Sur l'inégalité de fréquence dans l'isolement de *Yersinia enterocolitica* de part et d'autre de la frontière Franco-Belge. *Méd Mal Infect* **6**: 102-107.
302. **Mollaret HH, Bercovier H, and Alonso JM.** 1979. Summary of the data received at the WHO Reference Centre for *Yersinia enterocolitica*. *Contr Microbiol Immunol* **5**: 174-184.
303. **Morris K, and Feeley JC.** 1976. *Yersinia enterocolitica*: a review of its role in food hygiene. *WHO Bull* **54**: 79-85.
304. **Mousing J, Kyrval J, Jensen TK, Aalbæk B, Buttenschøn J, Svensmark B, and Willeberg P.** 1997. Meat safety consequences of implementing visual postmortem meat inspection procedures in Danish slaughter pigs. *Vet Rec* **140**: 472-477.
305. **Munk Petersen A, Vinther Nielsen S, Meyer D, Ganer P, and Ladefoged K.** 1996. Bacterial gastroenteritis among hospitalized patients in a Danish county. *Scand J Gastroenterol* **31**: 906-911.
306. **Nagamachi E, Shibuya S, Hirai Y, Matsushita O, Tomochika K, and Kanemasa Y.** 1991. Adapational changes of fatty acid composition and the physical state of membrane lipid following the change of growth temperature in *Yersinia enterocolitica*. *Microbiol Immunol* **35**: 1085-1093.
307. **Najdenski H, Iteman I, and Carniel E.** 1994. Efficient subtyping of pathogenic *Yersinia enterocolitica* strains by pulsed-field gel electrophoresis. *J Clin Microbiol* **32**: 2913-2920.
308. **Nakajima H, Inoue M, Mori T, Itoh K, Arakawa E, and Watanabe H.** 1992. Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. *J Clin Microbiol* **30**: 2484-2486.
309. **Necrasova LE, Aikimbaev AM, Meka-Mechenko TV, Atshabar BB, Burdelov SA, Meka-Mechenko VG, Dernovoi AG, Dernovaya VF, and Burdelov LA.** 1998a. Role of *Rattus norvegicus* in the epidemiology of yersiniosis in Almaty and its suburbs. Abstracts of the 7th Int Congress on

Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S45.

310. **Necrasova LE, Meka-Mechenko TV, Dmitrovsky AM, Atshabar BB, Dernovaya VG, Stepanov VM, and Bezrukova LS.** 1998b. Etiologic structure of yersiniosis in Almaty. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol SuppII* **6**: S45.
311. **Neogi PKB, Shahid NS, and Sanyal SC.** 1985. *Yersinia enterocolitica* infection: a case report from Bangladesh. *Trop Geogr Med* **37**: 362-364.
312. **Nesbakken T.** 1988. Enumeration of *Yersinia enterocolitica* O:3 from the porcine oral cavity, and its occurrence on cut surfaces of pig carcasses and the environment in a slaughterhouse. *Int J Food Microbiol* **6**: 287-293.
313. **Nesbakken T, Kapperud G, Sorum H, and Dommarsnes K.** 1987. Structural variability of 40-50 MDal virulence plasmids from *Yersinia enterocolitica*. *Acta Path Microbiol Immunol Scand Sect B* **95**: 167-173.
314. **Nesbakken T, Kapperud G, Dommarsnes K, Skurnik M, and Hornes E.** 1991. Comparative study of a DNA hybridization method and two isolation procedures for detection of *Yersinia enterocolitica* O:3 in naturally contaminated pork products. *Appl Environm Microbiol* **57**: 389-394.
315. **Nesbakken T, Kapperud G, Lassen J., and Skjerve E.** 1991. *Yersinia enterocolitica* O:3 antibodies in slaughterhouse employees, veterinarians, and military recruits: Occupational exposure to pigs as a risk factor for Yersiniosis. *Contr Microbiol Immunol* **12**: 32-39.
316. **Nesbakken T, Nerbrink E, Røtterud OJ, and Borch E.** 1994. Reduction of *Yersinia enterocolitica* and *Listeria* spp. on pig carcasses by enclosure of the rectum during slaughter. *Int J Food Microbiol* **23**: 197-208.
317. **Nesbakken T, and Skjerve E.** 1996. Interruption of microbial cycles in farm animals from farm to table. *Meat Sci* **43**, suppl: S47-S57.
318. **Nesbakken T, and Skjerve E.** 1998. Control of *Yersinia enterocolitica* in pigs at herd level and in the slaughterhouse. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S15.
319. **Neumeister B, Koerner K, Schwalbe B, and Kubanek B.** 1997. Fatal *Yersinia enterocolitica* septicemia after transfusion of red cells - case report and review of the literature. Infusionsther *Transfusionsmed* **24**: 14-19.
320. **Nielsen B, and Wegener HC.** 1997. Public health and pork products: regional perspectives of Denmark. *Rev Sci Tech Off Int Epizoot* **16**: 513-524.
321. **Niemie KM, Hannuksela M, and Salo OP.** 1976. Skin lesions in human yersiniosis. A histopathological and immunohistological study. *Br J Dermatol* **94**: 155-160.
322. **Niléhn B.** 1969. Studies on *Yersinia enterocolitica* with special reference to bacterial diagnosis and occurrence in human acute diarrhoeal disease. *Acta Pathol Microbiol Scand Suppl* **206**: 1-48.
323. **Nilsson A, Thisted Lambertz S, Stalhandske P, Norberg P, and Danielsson-Tham ML.** 1998. Detection of *Yersinia enterocolitica* in food by PCR amplification. *Lett Appl microbiol* **26**: 140-144.
324. **Noble MA, Barteluk RL, Freeman HJ, Subramaniam J, and Hudson JB.** 1987. Clinical significance of virulence-related assay of *Yersinia* species. *J Clin Microbiol* **25**: 802-807.
325. **Norberg P.** 1981. Enteropathogenic bacteria in frozen chicken. *Appl Env Microbiol* **42**: 32-34.
326. **Odinot PT, Meis JFGM, van den Hurk PJJC, Hoogkamp-Korstanje JAA, and Melchers WJG.** 1995. PCR-based DNA fingerprinting discriminates between different biotypes of *Yersinia enterocolitica*. *Contr Microbiol Immunol* **13**: 93-98.
327. **Ohtomo Y, Toyokawa Y, Saito M, Yamaguchi M, Kaneko S, and Maruyama T.** 1995. Epidemiology of *Yersinia enterocolitica* serovar O:8 infection in the Tsugaru area in Japan. *Contr Microbiol Immunol* **13**: 48-50.
328. **Olšovský Ž, Olšáková S, Chobot S, and Sviridov V.** 1975. Mass occurrence of *Yersinia enterocolitica* in two establishments of collective care of children. *J Hyg Epidem Microbiol Immun* **19**: 22-39.
329. **Olsvik O, and Kapperud G.** 1982. Enterotoxin production in milk at 22 and 4°C by *Escherichia coli* and *Yersinia enterocolitica*. *Appl Environ Microbiol* **43**: 997-1000.

330. Ossel C van, and Wauters G. 1990. Asymptomatic *Yersinia enterocolitica* infections during an outbreak in a day nursery. *Eur J Clin microbiol Infect Dis* **9**: 148.
331. Ostroff SM, Kapperud G, Hutwagner LC, Nesbakken T, Bean NH, Lassen J, and Tauxe RV. 1994. Sources of sporadic *Yersinia enterocolitica* infections in Norway: A prospective case-control study. *Epidemiol Infect* **112**: 133-141.
332. Ostroff SM. 1995. *Yersinia* as an emerging infection: epidemiologic aspects of Yersiniosis. *Contr Microbiol Immunol* **13**: 5-10.
333. Özbaz ZY, and Aytaç SA. 1993. Incidence of *Yersinia enterocolitica* in samples of pasteurized milk. *Chemie Mikrobiol Technol Lebensm* **15**: 129-133.
334. Paerregaard A, Shand GH, Gaarslev K, and Espersen F. 1991. Comparison of crossed immunoelectrophoresis, enzyme linked immunosorbent assays, and tube agglutination for serodiagnosis of *Yersinia enterocolitica* serotype O:3 infection. *J Clin Microbiol* **29**: 302-309.
335. Pai CH, Mors V, Toma S. 1978. Prevalence of enterotoxigenicity in human and non-human isolates of *Yersinia enterocolitica*. *Infect Immun* **22**: 334-338.
336. Pai CH, and DeStephano LC. 1982. Serum resistance associated with virulence in *Yersinia enterocolitica*. *Infect Immun* **35**: 605-611.
337. Paučková V, Kočí V, and Fukalová A. 1969. *Yersinia enterocolitica* in human faeces. *Zbl Bakt I Abt Orig* **209**: 504-509.
338. Pee W van, and Stragier J. 1979. Evaluation of some cold enrichment and isolation media for the recovery of *Yersinia enterocolitica*. *Antonie van Leeuwenhoek J Microbiol Ser* **45**: 465-477.
339. Persson C, Nordfelth R, Holmström A, Håkansson S, Rosqvist R, and Wolf-Watz H. 1995. Cell-surface-bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. *Mol Microbiol* **18**: 135-150.
340. Petrus M, Micheau P, Danjoux MF, Thore B, Rhabbour M, Moulie N, Bildstein G, and Netter JC. 1997. Succession arthrite purulente puis réactionelle à *Yersinia enterocolitica* chez un garçon de 8 ans. *Sem Hôp Paris* **73**: 286-288.
341. Piroth L, Meyer P, Bielefeld P, and Besancenot JF. 1997. Bactériémie à *Yersinia* et surcharge en fer. *Rev Méd Interne* **18**: 932-938.
342. Pramanik AK, Bhattacharyya HM, Chatterjee A, and Sengupta DN. 1980. Isolation of *Yersinia enterocolitica* from human and animal sources in the same family. *Ind J Animal Health* **19**: 79-81.
343. Prentice M. 1992. Transfusion *Yersinia enterocolitica* - rare but deadly. *Br Med J* **305**: 663-664.
344. Prentice MB, Cope D, and Swann RA. 1991. The epidemiology of *Yersinia enterocolitica* infection in the British Isles (1983-1988). *Contr Microbiol Immunol* **12**: 83-87.
345. Prpic JK, Robins-Browne RM, and Davey RB. 1983. Differentiation between virulent and avirulent *Yersinia enterocolitica* isolates by using Congo red agar. *J Clin Microbiol* **18**: 486-490.
346. Prpic JK, Robins-Browne RM, and Davey RB. 1985. In vitro assessment of virulence in *Yersinia enterocolitica* and related species. *J Clin Microbiol* **22**: 105-110.
347. Rabson AR, and Koornhof HJ. 1973. *Yersinia enterocolitica* infections in South Africa. *Contr Microbiol Immunol* **2**: 102-105.
348. Rakovský J, Paučková V, and Aldová E. 1973. Human *Yersinia enterocolitica* infections in Czechoslovakia. *Contr Microbiol Immunol* **2**: 93-98.
349. Ram S, Khurana S, Singh R, Sharma S, and Vadehra DV. 1987. *Yersinia enterocolitica* diarrhoea in north India. *Indian J Med Res* **86**: 9-13.
350. Ratnam S, Mercer E, Picco B, Parsons S, and Butler R. 1982. A nosocomial outbreak of diarrhoeal disease due to *Yersinia enterocolitica* serotype O:5, biotype 1. *J Infect Dis* **145**: 242-247.
351. Rea MC, Cogan TM, and Tobin S. 1992. Incidence of pathogenic bacteria in raw milk in Ireland. *J Appl Bacteriol* **73**: 331-336.
352. Robins-Browne RM, Rabson AR, and Koornhof HJ. 1973. *Yersinia enterocolitica* in South Africa. In: *Yersinia enterocolitica*. Bottone EJ (ed), CRC Press , Boca Raton, Florida, USA, pp 193-203.
353. Robins-Browne RM, Prpic JK, Stuart SJ. 1987. *Yersiniae* and iron. *Contr Microbiol Immunol* **9**: 254-258.

354. **Rohrbach BW, Draughon FA, Davidson PM, and Oliver SP.** 1992. Prevalence of *Listeria monocytogenes, Campylobacter jejuni, Yersinia enterocolitica* and *Salmonella* in bulk tank milk: risk factors and risk of human exposure. *J Food Prot* **55**: 93-97.
355. **Rosqvist R, Magnusson KE, and Wolf-Watz H.** 1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. *EMBO J* **13**: 964-972.
356. **Rüssmann H, Ruckdeschel K, and Heesemann J.** 1996. Translocation of *Yersinia enterocolitica* through an endothelial monolayer by polymorphonuclear leucocytes. *Infect Immun* **64**: 1016-1019.
357. **Rusu V, Lucinescu S, Stanescu C, Totescu E, and Muscan A.** 1973. Clinical and epidemiological aspects of the human *Yersinia enterocolitica* infections in Romania. *Contr Microbiol Immunol* **2**: 126-127.
358. **Saide-Albornoz JJ, Knipe CL, Murano EA, and Beran GW.** 1995. Contamination of pork carcasses during slaughter, fabrication, and chilled storage. *J Food Prot* **58**: 993-997.
359. **Samadi AR, Wachsmuth K, Huq MI, MahbubM, and Agbonlahor DE.** 1982. An attempt to detect *Yersinia enterocolitica* infection in Dhaka, Bangladesh. *Trop Geogr Med* **34**: 151-154.
360. **Sammarco ML, Ripabelli G, Ruberto A, Iannitto G, and Grasso GM.** 1997. Prevalence of Salmonellae, Listeriae and Yersiniae in the slaughterhouse environment and on work surfaces, equipment and workers. *J Food Prot* **60**: 367-371.
361. **Samonis G, Maraki S, Christidou A, Georgiladakis A, and Tselentis Y.** 1997. Bacterial pathogens associated with diarrhoea on the island of Crete. *Eur J Epidemiol* **13**: 831-836.
362. **Sandery M, Stinear T, and Kaucner C.** 1996. Detection of pathogenic *Yersinia enterocolitica* in environmental waters by PCR. *J Appl Bacteriol* **80**: 327-332.
363. **Sarigiannidou D, Koidis P, Batzios C, and Mantis A.** 1997. Behaviour of *Yersinia enterocolitica* in Mizithra-cheese. *Arch Lebensmittelhyg* **48**: 109-110.
364. **Schiemann DA, and Devenish JA.** 1982. Relationship of HeLa cell infectivity to biochemical, serological and virulence characteristics of *Yersinia enterocolitica*. *Infect Immun* **35**: 497-506.
365. **Schleifstein JI, and Coleman MB.** 1939. An unidentified microorganism resembling *B. lignieri* and *Pasteurella pseudotuberculosis*, and pathogenic for man. *NY State J Med* **39**: 1749-1753.
366. **Schleifstein JI, and Coleman MB.** 1943. *Bacterium enterocoliticum*. *NY State Dep Health, Ann Rep Divis Labor Res* **XX**: 56.
367. **Seuri M, and Granfors K.** 1992. Antibodies against *Yersinia* among farmers and slaughterhouse workers. *Scand J Work Environ Health* **18**: 128-132.
368. **Shayegani M, Maupin PS, and Waring A.** 1995. Prevalence and molecular typing of two pathogenic serogroups of *Yersinia enterocolitica* in New York State. *Contr Microbiol Immunol* **13**: 33-38.
369. **Shayegani M, Morse D, DeForge I, Root T, Malmberg-Parsons LM, and Maupin PS.** 1983. Microbiology of a major foodborne outbreak of gastroenteritis caused by *Yersinia enterocolitica* serogroup O:8. *J Clin Microbiol* **17**: 35-40.
370. **Shayegani M, and Parsons LM.** 1987. Epidemiology and pathogenicity of *Yersinia enterocolitica* in New York State. *Contr Microbiol Immunol* **9**: 41-47.
371. **Shenoy K, and Murano EA.** 1996. Effect of storage conditions on growth of heat-stressed *Yersinia enterocolitica* in ground pork. *J Food Protect* **59**: 365-369.
372. **Sheridan JJ, Logue CM, McDowell DA, Blair IS, Hegarty T, and Toivanen P.** 1998. The use of a surface adhesion immunoflurescent (SAIF) method for the rapid detection of *Yersinia enterocolitica* serotype O:3 in meat. *J Appl Microbiol* **85**: 737-745.
373. **Shiozawa K, Akiyama M, Sahara K, Hayashi M, Nishina T, Murakami M, and Asakawa Y.** 1987. Pathogenicity of *Yersinia enterocolitica* biotype 3B and 4, serotype O:3 isolates from pork samples and humans. *Contr Microbiol Immunol* **9**: 30-40.
374. **Shiozawa K, Nishina T, Miwa Y, Mori T, Akahane S, and Ito K.** 1991. Colonization in the tonsils of swine by *Yersinia enterocolitica*. *Contr Microbiol Immunol* **12**: 63-67.
375. **Shmilovitz M, and Kretzer B.** 1978. Isolates of *Yersinia enterocolitica* from clinical cases in northern Israel. *Isr J Med Sci* **14**: 1048-1055.
376. **Shorter NA, Thompson MD, Mooney DP, and Modlin JF.** 1998. Surgical aspects of an outbreak

of *Yersinia* enterocolitis. *Pediatr Surg Int* **13**: 2-5.
377. SIIDC (Swedish Institute for Infectious Diseases Control. 1997. Bacteriological diagnosis from the laboratory reports. Annual Report 1997, Department of Epidemiology, Table 13.
378. Sims GR, Glenister DA, Brocklehurst TF, and Lund BM. 1989. Survival and growth of poisoning bacteria following inoculation into Cottage cheese varieties. *Int J Food Microbiol* **9**: 173-195.
379. Singh G, Arora NK, Bhan MK, Ghai OP, Dhar S, and Shriniwas. 1983. Role of *Yersinia enterocolitica* in childhood diarrhoea. *Ind J Pediat* **50**: 39-42.
380. Slavchev G. 1989. (In Bulgarian) <Development and survival of *Yersinia enterocolitica* in butter.> *Vet Sbirka* **87**: 50-52.
381. Solomon T. 1995. Alexandre Yersin and the plague bacillus. *J Trop Med Hyg* **98**: 209-212.
382. Soltan Dallal MM, and Chitsaz M. 1998. Isolation of *Yersinia enterocolitica* from paediatric diarrhoea in Iran. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S37.
383. Sonnenwirth AC. 1968. Bacteraemia with and without meningitis due to *Yersinia enterocolitica, Comamonas terrigena, Edwardsiella tarda* and *Pseudomonas maltophilia*. *Ann NY Acad Sci* **174**: 488-502.
384. Staatz G, Alzen G, and Heimann G. 1998. Darminfektion, die häufigste Invaginationsursache im Kindesalter: Ergebnisse einer 10jährigen klinischen Studie. *Klin Pädiatr* **210**: 61-64.
385. Stefanov I. 1998. Isolation of *Yersiniae* from wild swine tonsils. Abstracts of the 7th Int Congress on Yersinia. *Ned Tijds Med Microbiol Supp II* **6**: S46.
386. Stenhouse MAE, and Milner LV. 1982. *Yersinia enterocolitica*: a hazard in blood transfusion. *Transfusion* **22**: 396-398.
387. Stern NJ, Pierson MD, and Kotula AW. 1980. Effects of pH and sodiumchloride on *Yersinia enterocolitica* growth at room and refrigeration temperature. *J Food Sci* **45**: 64-67.
388. Stern NJ, Pierson MD, and Kotula AW. 1980. Growth and competitive nature of *Yersinia enterocolitica* in whole milk. *J Food Sci* **45**: 972-974.
389. Stolk-Engelaar VMM, and Hoogkamp-Korstanje JAA. 1996. Clinical presentation and diagnosis of gastrointestinal infections by *Yersinia enterocolitica* in 261 Dutch patients. *Scand J Infect Dis* **28**: 571-575.
390. Straley SC, and Perry RD. 1995. Environmental modulation of gene expression and pathogenesis in *Yersinia*. *Trends Microbiol* **3**: 310-317.
391. Sulakvelidze A, Dalakishvili K, Barry E, Wauters G, Robins-Browne R, Imnadze P, and Glenn Morris Jr. J. 1996. Analysis of clinical and environmental *Yersinia* isolates in the Republic of Georgia. *J Clin Microbiol* **34**: 2325-2327.
392. Sulakvelidze A, Saillour M, Glenn Morris Jr. J, Ronco E, Perrone C, de Truchis P, and Nordmann P. 1998. Isolation of *Yersinia enterocolitica* from HIV-positive patients with diarrhoea: "nonpathogenic" strains as possible cause. *Infect Dis Clin Practice* **7**: 424-426.
393. Svanteson B, Thorén A, Castor B, Barkenius G, Bergdahl U, Tufvesson B, Hansson HB, Möllby R, and Juhlin I. 1988. Acute diarrhoea in adults: aetiology, clinical appearance and therapeutic aspects. *Scan J Infect Dis* **20**: 303-314.
394. Szita J, Káli M, and Rédey B. 1973. Incidence of *Y. enterocolitica* infection in Hungary. *Contr Microbiol Immunol* **2**: 106-110.
395. Taccetti G, Trapani S, Ermini M, and Falcini F. 1994. Reactive arthritis triggered by *Yersinia enterocolitica*: a review of 18 pediatric cases. *Clin Exp Rheum* **12**: 681-684.
396. Tacket CO, Narain JP, Sattin R, Lofgren JP, Konigsberg jr. C, Rendtorff RC, Rausa A, Davis BR, and Cohen ML. 1984. A multistate outbreak of infections caused by *Yersinia enterocolitica* transmitted by pasteurized milk. *JAMA* **251**: 483-486.
397. Tacket CO, Ballard J, Harris H, Allard J, Nolan C, Quan T, and Cohen ML. 1985. An outbreak of *Yersinia enterocolitica* infections caused by contaminated tofu (soybean curd). *Am J Epidemiol* **121**: 705-711.
398. Tamburrino V, Monno R, Valenza MA, and Numo R. 1993. Incidence of *Yersinia enterocolitica* antibodies in patients with inflammatory joint diseases. *Clin Rheum* **12**: 354-356.
399. Tame S, Wit de D, and Meek A. 1998. *Yersinia enterocolitica* and mycotic aneurysm. *Aust NZ*

J Surg **68**: 813-814.

400. Tauxe RV. 1997. Emerging foodborne diseases: an evolving public health challenge. *Emerg Infect Dis* **3**: 425-434.

401. Tauxe RV, Wauters G, Goossens G, Van Noyen R, Vandepitte J, Martin SM, De Mol P, and Thiers G. 1987. *Yersinia enterocolitica* infections and pork: The missing link. *Lancet i*: 1129-1132.

402. Theakston EP, Morris AJ, Streat SJ, Baker BW, and Woodfield DG. 1997. Transfusion transmitted *Yersinia enterocolitica* infection in New Zealand. *Aust NZ J Med* **27**: 62-67.

403. Thisted Lambertz S, Ballagi-Pordáni A, Nilsson A, Norberg P, and Danielsson-Tham ML. 1996. A comparison between a PCR method and a conventional culture method for detecting pathogenic *Yersinia enterocolitica* in food. *J Appl Bacteriol* **81**: 303-308.

404. Tipple MA, Bland LA, Murphy JJ, *et al.*. 1990. Sepsis associated with transfusion of red cells contaminated with *Yersinia enterocolitica*. *Transfusion* **30**: 207-213.

405. Toivanen P, Toivanen A, Olkkonen L, and Aantaa S. 1973. Hospital outbreak of *Yersinia enterocolitica* infection. *Lancet i*: 801-803.

406. Toma S, Lafleur L, and Deidrick VR. 1979. Canadian experience with *Yersinia enterocolitica* (1966-1977). *Contr Microbiol Immunol* **5**: 144-149.

407. Trebesius K, Harmsen D, Rakin A, Schmelz J, and Heesemann J. 1998. Development of rRNA-targeted PCR and in situ hybridization with fluorescently labelled oligonucleotides for detection of *Yersinia* species. *J Clin Microbiol* **36**: 2557-2564.

408. Tsai SJ, and Chen LH. 1991. Occurrence of *Yersinia enterocolitica* in pork products from Northern Taiwan. *Contr Microbiol Immunol* **12**: 56-62.

409. Turkson PK, Lindqvist KJ, and Kapperud G. Isolation of *Campylobacter* spp. and *Yersinia enterocolitica* from domestic animals and human patients in Kenya. *APMIS* **96**: 141-146.

410. Uhnoo I, Wadell G, Svensson L, Olding-Stenkvist, Ekwall E, and Mölby R. 1986. Aetiology and epidemiology of acute gastro-enteritis in Swedish children. *J Infect* **13**: 73-89.

411. Une T. 1977. Studies on the pathogenicity of *Yersinia enterocolitica*. II Interaction with cultured cells in vitro. *Microbiol Immunol* **21**: 365-377.

412. Ursing J, Brenner DJ, Bercovier H, Fanning GR, Steigerwalt AG, Brault J, and Mollaret HH. 1980. *Yersinia frederiksenii*: a new species of Enterobacteriaceae composed of rhamnose-positive strains (formerly called *Yersinia enterocolitica* or *Yersinia enterocolitica*-like). *Curr Microbiol* **4**: 213-217.

413. Vandepitte J, Wauters G, and Isebaert A. 1973. Epidemiology of *Yersinia enterocolitica* infections in Belgium. *Contr Microbiol Immunol* **2**: 111-119.

414. Van Noyen R, Selderslaghs R, Wauters G, and Vandepitte J. 1987a. Comparative epidemiology of *Yersinia enterocolitica* and related species in patients and healthy controls. *Contr Microbiol Immunol* **9**: 61-67.

415. Van Noyen R, Selderslaghs R, Wauters G, and Vandepitte J. 1987b. Optimal recovery of *Yersinia enterocolitica* O:3 and O:9 from stools of patients with intestinal disorders. *Contr Microbiol Immunol* **9**: 272-278.

416. Van Noyen R, Selderslaghs R, Bogaerts A, Verhaegen J, and Wauters G. 1995. *Yersinia pseudotuberculosis* in stool from patients in a regional Belgian hospital. *Contr Microbiol Immunol* **13**: 19-24.

417. Varghese A, Ramachandran VG, and Agarwal DS. 1984. Isolation and characterization of *Yersinia enterocolitica* from patients of gastroenteritis. *Ind J Med Res* **79**: 35-40.

418. Velázquez LC del, Escudero ME, and de Guzmán AMS 1993. Biovars, serovars and phagovars of *Yersinia enterocolitica* isolated from 450 samples of cold food in San Luis, Argentina. *J Food Prot* **56**: 333-335.

419. Velázquez LC del, Escudero ME, and de Guzmán AMS 1996. Prevalence of *Yersinia enterocolitica* in Hake (*Merluccius hubbsi*) fillets. *J Food Prot* **59**: 781-783.

420. Verhaegen J, Dancsa L, Lemmens P, Janssens M, Verbist L, Vandepitte J, and Wauters G. 1991. *Yersinia enterocolitica* surveillance in Belgium (1979-1989). *Contr Microbiol Immunol* **12**: 11-16.

421. **Verhaegen J, Charlier J, Lemmens P, Delmée M, Noyen R van, Verbist L, and Wauters G.** 1998. Surveillance of human *Yersinia enterocolitica* infections in Belgium: 1967-1996. *Clin Infect Dis* **27**: 59-64.
422. **Viitanen AM, Arstila TP, Lahesmaa R, Granfors K, Skurnik M, and Toivanen P.** 1991. Application of the polymerase chain reaction and immunofluorescence techniques to the detection of bacteria in *Yersinia*-triggered reactive arthritis. *Arthr Rheum* **34**: 89-96.
423. **Wachsmuth K, Kay BA, Birkness KA.** 1984. Diagnostic value of plasmid analysis and assays for virulence in *Yersinia enterocolitica*. *Diagn Microbiol Infect Dis* **2**: 219-228.
424. **Wauters G.** 1973. Improved methods for the isolation and the recognition of *Yersinia enterocolitica*. *Contr Microbiol Immunol* **2**: 68-70.
425. **Wauters G.** 1981. Antigens of *Yersinia enterocolitica*. In:*Yersinia enterocolitica*. Bottone EJ (ed), CRC Press Inc, Boca Raton, Florida, USA, pp: 41-53.
426. **Wauters G, Aleksić S, Charlier J, and Schulze G.** 1991. Somatic and flagellar antigens of *Yersinia enterocolitica* and related species. *Contrib Microbiol Immunol* 12: 239-243.
427. **Wauters G, Janssens M, Steigerwalt AG, and Brenner DJ.** 1988a. *Yersinia mollaretii* sp.nov. and *Yersinia bercovieri* sp. nov., formerly called *Yersinia enterocolitica* biogroups 3A and 3B. *Int J Syst Bact* **38**: 424-429.
428. **Wauters G, Goossens V, Janssens M, and Vandepitte J.** 1988b. New enrichment method for isolation of pathogenic *Yersinia enterocolitica* serogroup O:3 from pork. *Appl Environ Microbiol* **54**: 851-854.
429. **Weir WRC.** 1985. Bacterial infections: *Yersinia* infection. *Curr Opin Gastroenterol* **1**: 135-137.
430. **Weissfeld AS, and Sonnenwirth AC.** 1980. *Yersinia enterocolitica* in adults with gastrointestinal disturbances: need for cold enrichment. *J Clin Microbiol* **11**: 196-197.
431. **Wenzel BE, Peters A, and Zubaschev I.** 1996. Bacterial virulence antigens and the pathogenesis of autoimmune thyroid diseases (AITD). *Exp Clin Endocrinol Diabetes* **104** Suppl 4: 75-78.
432. **Wetzler TF, and Hubbert WT.** 1968. *Yersinia enterocolitica* in North America. Int Symp Pseudotuberculosis, Paris 1967. Karger, Basel/New York. *Symp Ser Immunobiol Stand*, Vol 9: 343-356.
433. **Weynants V, Jadot V, Denoel PA, Tibor A, and Letesson JJ.** 1996. Detection of *Yersinia enterocolitica* serogroup O:3 by a PCR method. *J Clin Microbiol* **34**: 1224-1227.
434. **WHO.** 1981. Yersiniosis. World Health Organization Regional Office for Europe, Copenhagen, Denmark. *EURO Rep Stud* **60**.
435. **Wilson HD, McCormick JB, and Feeley JC.** 1976. *Yersinia enterocolitica* infection in a 4-month-old infant associated with infection in household dogs. *J Pediatr* **89**: 767-769.
436. **Winblad S.** 1967. Studies on serological typing of *Yersinia enterocolitica Acta Pathol Microbiol Scand Suppl* **187**: 115.
437. **Winblad S.** 1973. The clinical panorama of human *Yersiniosis enterocolitica*. *Contr Microbiol Immunol* **2**: 129-132.
438. **Winblad S, Niléhn B, and Sternby NH.** 1966. *Yersinia enterocolitica* (*Pasteurella X*) in human enteric infections. *Brit Med J ii*: 1363-1366.
439. **Wit MAS de, Kortbeek LM, van Leeuwen WJ, Koopmans MPG, Bartelds AIM, van Asperen IA, and Borgdorff MW.** 1997. *Interimrapportage van onderzoek naar gastro-enteritis in huisartsen-peilstations (NIVEL) 1996-1997.* (In Dutch) <Interim report of a study into gastroenteritis in sentinel practices in the Netherlands.> National Institute of Public Health and the Environment, Bilthoven, The Netherlands. *RIVM report* nr. 216852001.
440. **Wren BW, and Tabaqchali S.** 1990. Detection of pathogenic *Yersinia enterocolitica* by the polymerase chain reaction. *Lancet* **336**: 693.
441. **Wulf CJMA de, Esseveld H, and Goudzwaard C.** 1969. Infectie door *Yersinia enterocolitica* bij de mens met karakteristieke ontsteking in de ileo-coecaalstreek. *(In Dutch)* <*Yersinia enterocolitica* infection in a human patient with characteristic inflammation in the ileo-coecal area.> *Ned T Geneesk* **113**: 665-667.
442. **Wuthe HH, Aleksić S, and Kwapil S.** 1995. *Yersinia* in the European Brown hare of Northern Germany. *Contr Microbiol Immunol* **13**: 51-54.

443. Zen-Yoji H. 1981 Epidemiologic aspects of Yersiniosis in Japan. In:*Yersinia enterocolitica*. Bottone EJ (ed) CRC Press Inc, Boca Raton, Florida, USA, pp 205-216.

444. Zen-Yoji H, and Maruyama T. 1972. The first successful isolations and identification of *Yersinia enterocolitica* from human case in Japan. *Jpn J Microbiol* **16**: 493-500.

445. Zheng XB, and Xie C. 1996. Isolation, characterization and epidemiology of *Yersinia enterocolitica* from humans and animals. *J Appl Bacteriol* **81**: 681-684.

2

The Cold Chain

food preservation in historical perspective

ABSTRACT

Refrigeration, athough not a recent discovery, has, in our era, become a general means of keeping foods from spoilage at all stages between production and consumption. However, chilling is not enough to restrain certain micro-organisms, i.e. those which are known as 'psychrophiles' or 'psychrotrophs' because of their ability to muliplicate at temperatures near zero. Among these are not only many spoilage bacteria, such as *Pseudomonas* spp., but also several bacteria that are pathogenic for humans, especially *Yersinia enterocolitica* and *Listeria monocytogenes*. Infection with these latter organisms has emerged as an important cause of foodborne disease in the last four decades. This undesirable development has presumably been favoured by the enormous increases that have occurred in the application of refrigeration for the preservation of perishable foods. In addition, refrigeration is not without danger in modern medical technology. Rare, but not to be neglected, are fatalities which occur in blood transfusions, due to bacterial growth during extended chilled storage of the administered blood-products. *Yersinia entrocolitica* is the major organism involved in this latter type of infection.

2.1 TRADITIONAL METHODS OF FOOD PRESERVATION

2.1.1 'Natural' Methods: Drying and Freezing

When life was largely nomadic, food preservation was not under discussion because man literally lived from hand to mouth: food was gathered and eaten where and when it could be found. But when life became sedentary, approximately 10,000 years ago, keeping some of the harvest bounty for use during the non-productive season became a vital necessity for survival [Baumgartner 1978]. Whilst our ancestors must have experienced that almost all raw foods are liable to spoilage at some rate or other, they will not have failed to notice too that the deterioration process was sometimes delayed by climatic factors [1].

Early man must have been aware that naturally dried products such as nuts and seeds hardly go bad: witness the storage-pits for dried grains that have been found in the valleys of the Indus and Euphrates-Tigris, the cradle of our civilization [Borgstrom 1968]. In extremely low temperature regions, on the other hand, early man will have noticed that captured fish or game could be preserved by freezing, just by hanging it outdoors. When learning, probably by chance, how to use utilize these factors, early man started to imitate nature. Thus, by just taking advantage of the sun's heat, wind and frosty cold, he (or she) 'invented' drying and freezing as the first methods of food preservation.

2.1.2 First 'Artificial' Methods: Smoking, Salting, Chilling

As time went on, other more or less natural resources, such as smoke, salt, snow and ice, were also adopted to keep food surpluses edible for longer periods. Whether or not they were combined with drying, new techniques such as smoking, salting, curing and fermenting became widely used for long-range preservation of highly perishable foods, such as meat, fish

1. Drying was most probably the first method adapted by man to enlarge the storage-life of food surpluses. Presumably, sun-dried seeds served as a model for solar drying of more perishable foodstuffs. Anyhow, dried foodstuffs have been used for centuries, all over the world: ranging from sun-dried fruits in ancient Mesopotamia and Palestine to dried, salted fish with the Phoenicians, and from freeze-dried potato tubers with the pre-Columbian Indians in Peru and Bolivia to sun- or wind-dried meat as the typical travelling food of hunting and migratory people from Central Asia, Africa and the American continent. Similarly, man must have learned by experience that foodstuffs also kept better in the cold. Prehistorians think that in the lower Old Stone Age (100,000 B.C.) caves, such as those in Altamira in Spain, were used for cool storage of food, especially game. Old written documents also testify that man was positively aware of the benefit of cool storage: in 40 A.D., the Latin agronomist Columella gives advice on the orientation of buildings so as to obtain a cool ambience for keeping cheese, fruit and vegetables. In Spain, around 1200, recommendations and prescribed rules concerning the cooling of carcasses after slaughter were given, and around 1600, summer storage of game in caves was recorded in alpine regions of Austria and Switzerland.

and milk. To keep less vulnerable products such as fruits, cabbages and tubers from precocious spoilage, man in general took advantage of natural cold in caves, cellars and cold springs [Jobse-van Putten 1989; Reinink & Vermeulen 1981; Plank 1960; Thévenot 1979]. In addition, snow and ice were sometimes used for cooling purposes, especially during long-distance transport of perishables such as fish and fruit [Legge 1876; Mez 1922; Forbes 1966; Tannahill 1988; Toussaint-Samat 1992][2]. This latter habit, however, mainly served the tables of the rich and was not used for common people's food supply. In general, chilling by ice played a minor role in food preservation.

2.1.3 Mechanisms of Effectiveness

For ages, the factual action of the empirically developed preservation methods was veiled in mysteries: when the Spanish doctor Nicolas Monardes reviews in 1574 the means of cooling of foodstuffs which were in use at that time, he sighs that the delaying effect of snow on the rotting of fruits, fish and meat is "... *something which human intelligence can neither understand nor explain...*" [Thévenot 1979]. In fact, the mechanisms of the effectiveness of preservation methods were not understood before the end of the 19th century, after Louis Pasteur in the 1860s had established the relationship between microbial activity and putrefaction.

Nevertheless, all the empirically developed methods to arrest or retard the natural processes of decay were based on sound microbiological principles. These were either suppression of unwanted bacterial growth, by reducing water activity and/or temperature, or, on the contrary, favouring the growth of advantageous types of organisms by adding them deliberately and/or providing an optimal environment (Table 2.1, upper part)

2. Food preservation by refrigeration hasn't just sprung up overnight. Almost four thousand years ago, snow and ice were harvested and stored for later use as refrigerants, as may be concluded from inscriptions on Mesopotamian clay tablets and from passages in the 'Shi-King', the classic Chinese 'Book of Poetry', in which Confucius recorded aspects of daily life in the period between 1800 and 1300 B.C. Born in the Far East, storage of snow and ice became widespread in the Old World, wherever cold winters or mountain altitudes permitted its harvest: ice-pits, -cellars and -houses have left their traces in Japan and India, as well as all over the Mediterranean and Black Sea regions.
In many cases, the snow and ice had to be carried down from distant mountains, and it was initially mainly used for luxury applications: cooling of wine and drinking water, as was common practice amongst the privileged people of ancient Rome, or preparation of fruit-sorbets, which were very popular in the Arab world of the Middle Ages.
But ice was also used as a preservative agent during food-transports in the hot season. The markets of ancient Rome, for example, were provided with fish from the Rhine and the North Sea, packed round with ice and transported under layers of insulating furs. Much later, in the 10th century, watermelons were sent from northern Iran to Baghdad in ice-cooled lead containers. And in the 16th century, plums and bamboo shoots were being transported to the palaces of the Chinese emperors in Peking by keeping them fresh in ice.

Table 2.1 Methods of food preservation, adapted from [Adams & Moss 1995]

TREATMENT	TEMP. RANGE	AIMED EFFECT
Drying, curing, and concentration	< 100°C	Inhibition of growth by reducing wateractivity
Fermentation, and addition of preservatives	RT*	Arrest of unwanted growth by addition of inhibitory factors
Freezing and chilling	-20 to 4°C	Arrest of growth by reducing metabolic activity
Sterilization	> 100°C	Complete elimination of viable micro-organisms
Cooking	± 100°C	Destruction of pathogenic micro-organisms
Pasteurization	60 - 80°C	Elimination of pathogens & spoilage organisms
Vacuum- & Modified-atmosphere-packaging	-20°C to RT	Arrest of growth by imposing of inhibitory factors
Ionizing radiation	-20°C to RT	Elimination of viable micro-organisms by damaging of DNA

* RT = room temperature

2.1.4 Nutritional Drawbacks

Just as people were unaware of microbiological principles, so the basics of wholesome food were misunderstood. Food was largely thought of as containing a single element to sustain life, and a possible decrease in nutritional value during extended storage was not under dis-cussion. The traditional methods of drying, smoking, salting, curing and fermenting stood undisputed for ages, in spite of their apparent drawbacks with respect to loss of attractive-ness and taste. Besides, common people relied heavily on such preserved foods, from sheer necessity. The resulting monotonous diets, which were usually short of essential nutrients, contributed greatly to reduced public health, but it took until the 1830s before the connec-tion between malnutrition and unhealthiness was recognized [Borgstrom 1968; Hartog 1980]. The alimentary situation, however, was not to be altered before a drastic reform had taken place in the field of food preservation.

2.2 REVOLUTIONS IN FOOD PRESERVATION

2.2.1 New Heat-treatments

With the onset of the industrial era, heat was discovered to be a useful tool to realize an increase in the storage life of perishable foodstuffs. Heat-preservation was invented by the French chef Nicolas Appert who described, in 1811, a procedure of boiling combined with

hermetic closure as the "... *Art of Conserving all kinds of Animal and Vegetable Matter for several Years*", by which canning was born [Adams & Moss 1995]. Initially, the success of this technique was erroneously ascribed to the exclusion of air from the product. It took fifty years more before Louis Pasteur recognized that microbial inactivation was in fact the essential event. He developed a milder heat-processing technique, by which harmful micro-organisms could be destroyed without deactivating the useful ones. When the mechanisms of effectiveness of 'appertization' and 'pasteurization' were established, their value in protecting public health was also recognized, and they became increasingly used in food preservation.

2.2.2 Revaluation of Cold

Ice-houses

From the 16th century onwards, cold became revalued as a useful 'additive' in the human diet. Strictly speaking, the application of cold was not new, since ice and snow had been used for the cooling of spices and drinks in ancient times (see also footnote 2). For ages, however, the icing of foods was unusual and the application of ice and snow had been mainly restricted to the cooling of wine and drinking water, in addition to use for nursing and air-conditioning(!) [Plank 1954]. A change started to set in when, in the 16th and 17th centuries, sorbets and ice-creams came into fashion in Europe and North-America [Tannahill 1988; Adams & Moss 1995]. By the 18th century, special ice-houses or -cellars had become a common feature at Europe's stately homes: huge amounts of ice were stored yearly, especially for the cooling of wine and the preparation of luxury refreshments [Reinink & Vermeulen 1981]. The preservative qualities of ice remained, of course, not unnoticed, but the ice-houses were as yet scarcely used for food preservation, since the well-to-do, in their situation of comfort, had little interest in preserved foods. Ordinary people, on the other hand, could not afford such stores, nor the labour-intensive harvesting of large quantities of ice. Only in Spain, where the very hot summers provoked early research into the benefits of natural cold, was trade in snow a public affair in those days, and refrigeration generally adapted to foods [Thévenot 1979]. Until the end of the 18th century, the icing of foods for preservation purposes was in most other countries a private and small scale phenomenon that was mainly restricted to the cooling of milk, butter and meat products on farms [Reinink & Vermeulen 1981].

The victory march of ice

At the end of the 18th century, almost simultaneously with the invention of heat-preservation, the application of cold as a preservative agent received a new impetus: the harvesting and storage of ice started to become rationalized, which meant that it came within the reach of common people. When, at the turn of the century, the collection of natural ice became further mechanized, ice-houses mushroomed at farms all over North America. Similar progress was made in the ways of transport and storage, a process that went hand in hand with

commercialization of the ice-trade, especially in the USA and Scandinavia (Figure 2.1)[3].

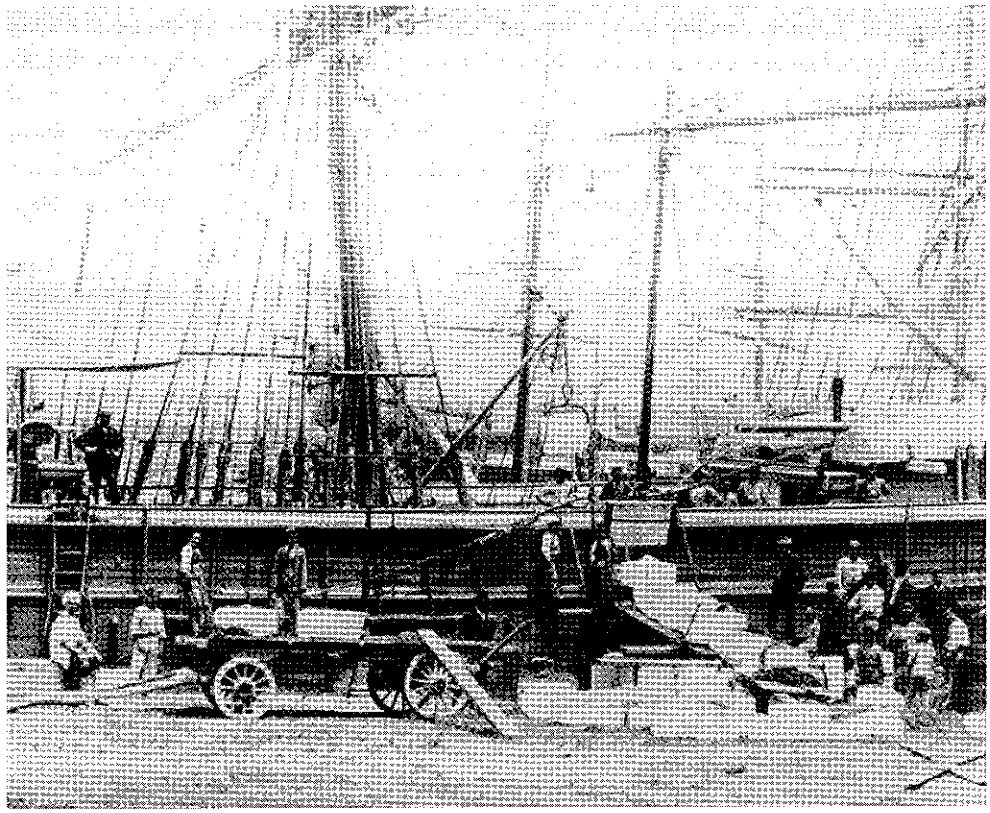


Figure 2.1 The sailing ship '*Tenax Propositi*' from Kragerø (Norway) in the harbour of Philippeville (Algeria), in 1886, delivering Norwegian ice. Copied from [Reinink & Vermeulen 1981], with permission.

3. The real breakthrough of refrigeration as a food-preservation method took place in the course of the 19th century, and its triumphal progress was initiated in North America and Europe. Here, the industrialization of harvesting, storage and transport of natural ice was realized, which was a prerequisite for the enormous expansion of ice-consumption that was, in its turn, the driving force for the development of mechanical refrigeration. Some figures may illustrate the scope of cooling by ice at the end of the 19th century: yearly, five million tonnes of ice were harvested in the United States, mainly for use in the meat- and fish-trade. Annually, some 8000 tonnes of fish from the North American Great Lakes was frozen in salt/ice mixtures, and 30,000 tonnes of iced beef were shipped from the United States to Great Britain. Private consumption also reached enormous proportions, as is evident from the 700 kg of ice that were used yearly per head by the citizens of a large town like New York. Some 200,000 tonnes of American ice were exported to destinations as far away as Rio de Janeiro, Calcutta and Australia, and the export of Norwegian ice to Great Britain and Germany was even larger, reaching levels of half a million tonnes per year.

These developments cleared the way for a more general utilization of ice in food preservation [Thévenot 1979]: a boom followed in ice-consumption and in the 1830s, the application of ice had become common practice in the meat- and fish-trade in both North America and Europe [Papworth 1832; Thévenot 1979; Jobse-van Putten 1996]. To meet the growing needs of the market, the ice-business was quickly industrialized, which mutually stimulated further expansion of refrigeration as a means of food preservation. When, halfway through the 19th century, special cooling cabinets for private use made their entry, the refrigeration of perishable foodstuffs became generally adopted in big towns, where it was distributed by doorstep deliveries at hotels, restaurants and well-to-do households (Figure 2.2).

Figure 2.2 Street scene in the Netherlands, in the 1930s: blocks of ice are delivered at a restaurant (*Fotoarchief Spaarnestad*)

Following the improvements in the meat- and fish-trade, large-scale application of refrigeration spread to other commodities: cold stores for apples were built, and ice-cooled as well as ice/salt-refrigerated rail transport of fruits and butter started in the last quarter of the 19th century [Plank 1954; Thévenot 1979].

Mechanization of refrigeration

Natural ice as a cooling medium, however, had many drawbacks, from which contamination during its formation and handling formed the main problem for direct application on foods. So much effort was devoted to realizing a cleaner (and less space-occupying) production of cold. In the second half of the 19th century, when the industrial revolution was in full swing, the principles of thermodynamics were established and a wide range of new mechanical devices for all kinds of techniques, including power supply, became available. Simultaneously, the utilization of liquefiable gases was mastered and before the turn of the century, mechanical refrigeration was a fact. Breweries, and ships for the international transport of meat, were the first to be equipped with refrigerating machines [Thévenot 1970; Plank 1962]. In the first decades of the 20th century, refrigeration machines were installed in dairy plants and in public cold stores, and the capacity of mechanically refrigerated transport expanded enormously.

2.2.3 Development of the 'Cold Chain'

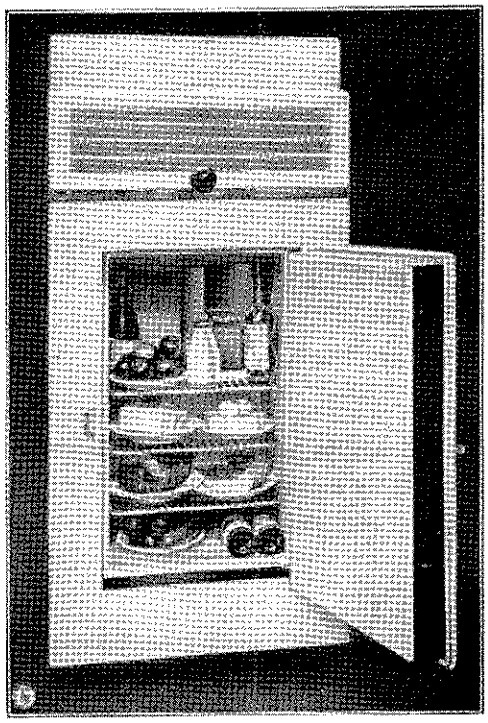


Figure 2.3 The 'Protos-Frigor': one of the first mechanical house-hold refrigerators in the Netherlands, (copied from *'Koeltechniek'*, 1932)

Although natural ice had paved the way, the mechanization of refrigeration was essential for the development of the 'Cold Chain'. In the 1930s, the world-wide commercial refrigeration of all types of foodstuffs grew in size, favoured by both internal and external factors, such as use of the newly developed freons and completion of the electricity grid in the industrialized countries. In the first half of the 20th century, mechanical refrigeration integrated also into the private sector. The household refrigerator was launched in 1918, spread in the USA in the Interbellum, and became current in the other industrialized countries after the second world war [Thénevot 1979; Plank 1962; Jobse-van Putten 1996].

The maturation of the 'Cold Chain' was beginning to take shape in the middle of the 20th century, when trade became more and more centralized and refrigerating equipment was incorporated in retail food

distribution systems in most developedcountries, a development that was speeded up by the introduction of supermarkets. When, finally in the 1960s, the household refrigerator had become an integral part of kitchen furniture in all industrialized countries, the ultimate link in the 'cold chain' was closed. Actually, a continuous cold chain from producer to consumer has evolved, and nowadays cooling is an indispensable factor in the commerce of foodstuffs [Gac 1992].

2.3 THE ALIMENTARY REVOLUTION

2.3.1 Healthier Diets

As a result of the changing nutritional insights, a clear concept of the superiority of fresh products prevailed throughout the last half of the 19th century and interest grew for cooling of various foodstuffs. Canning and pasteurization had already brought about a nutritional revolution, but the mechanization of refrigeration initiated a second, even more drastic breakthrough in food-handling and dietary patterns. First, the age-long dominant position of vegetable food in the ordinary man's diet was finally broken down when 'fresh' meat which everybody could afford became available: in fact, chilled and frozen meat imported from Argentina, Australia and New Zealand fed the rapidly increasing populations of Europe and North-America in the first decades of the 20th century. Second, large scale trans- and intercontinental transports enabled delivery of fresh fruits and vegetables for longer periods and to wider geographical areas, which consequently enlarged nutritional diversity, especially in the industrialized countries.

2.3.2 New Products and Changing Preferences

By the maturation of the 'cold chain' in the second half of our century, and the simultaneous expansion of the agro-alimentary industry, chilling and freezing became the keystones of the world food supply. As a result, our food and eating habits were changed more in the fifty years after the second world war than in the previous nineteen centuries [Fenton & Kisbán 1986; Otterlo 1990; Jobse-van Putten 1989].

Convenience foods

New commodities such as ready-to-eat meals and other convenience foods appeared upon the scene and, in addition, a tremendous growth was seen in the consumption of quick-frozen foods. In the last decades, stress has been laid not only on ease of preparation, but also on maximum retention of the original qualities of foodstuffs with respect to appearance, flavour, and dietetic values [Gould 1996]. Through the consumer's preference for foods that are more

convenient but at the same time less heavily processed and not loaded with additive preservatives, refrigeration has become even more important because it is, in general, the gentlest method of food preservation, having relatively few adverse effects upon taste, texture and nutritional value [Potter & Hotchkiss 1995].

Chilled products with extended shelf life

To meet the consumers demands for more 'natural' and fresh products, new food processing technologies have been developed to extend the shelf life of refrigerated products. These methods include mild heating, pH reduction, and vacuum- or modified atmosphere packaging, resulting in 'Sous-Vide'-products, and so-called REPFEDs (i.e. Refrigerated Pasteurized Foods of Extended Durability) [Lechowich 1988; Sofos 1993; Gould 1996]. To keep the microbial population of such foods under control, several relatively mild preservative factors are combined, creating a so-called hurdle effect [Leistner 1995; Gould 1996]. The hurdle technology is aimed at optimalization of the safety and quality of products by adjustment of the different preservative factors (= 'hurdles') applied. Nevertheless, evidence is growing that even the introduction of these new technologies may not completely expel the risks of food spoilage or food poisoning [Mossel & Struijk 1991; Notermans *et al.* 1990]. After all, a wide range of homeostatic mechanisms have evolved in micro-organisms, which enable them to survive various types of extreme environmental stresses. Exposure to one type of stress can induce resistance to other environmental extremes, the so-called Global Stress Response [Gould *et al.* 1995]. Some of these adaptations may be just the thing needed to leap one or more hurdles in modern food-preservation, and the ability to survive and proliferate at low temperatures might very well be one of these features.

2.4 RISKS OF COLD PRESERVATION

2.4.1 Cold-tolerant Food Spoilage Organisms

With the advent of the refrigeration era, the idea took form that refrigeration could probably be enough to keep foods from precocious spoilage. In 1936, for example, it was recommended that foods should be stored below 10°C and preferably at 4°C, "... *to prevent growth of pathogens or toxin development...*" [Prescott & Geer 1936]. By the mid-1950s, however, it became evident that cooling, though it indeed greatly retarded microbial growth and production of undesirable metabolites, could not completely prevent the growth of food spoilage organisms [Walker & Stringer 1990]. Various bacteria, yeasts and molds appeared to grow in a wide range of food products held at 5°C. Coincidental with improved refrigeration over the years, a change in the spoilage flora of many types of food occurred, and cold-tolerant organisms became an escalating problem for the food-industry [Tompkin 1973; Palumbo 1986; Sørhaug & Stepaniak 1997].

2.4.2 Cold-tolerant food-borne Pathogens

Cold-loving 'Psychrophiles' and 'Psychrotrophs'

From the 1950s onwards, it was taken into account that a decreased storage temperature only retards food spoilage, but refrigeration was still considered adequate to prevent growth and/or toxin formation by foodborne pathogens. In the early 1960s, however, it became evident that refrigeration is not enough to restrain foodborne pathogens, since several 'cold-loving' organisms, called psychrotrophs or psychrophiles, appeared to be amongst them. Since then, several human pathogenic bacteria have been shown to grow and/or produce toxins in foods held at 5°C or less, in particular *Listeria monocytogenes*, *Yersinia enterocolitica*, *Aeromonas hydrophyla* and certain types of *Clostridium botulinum* (Table 2.2) [Olsvik & Kapperud 1982].

Table 2.2 Psychrotrophic* bacteria involved in foodborne disease. Adapted from [Kraft 1992; Adams & Moss 1995]

ORGANISM	GRAM-TYPE	OXYGEN TOLERANCE	MOTILITY
Aeromonas hydrophyla	-	facultative	+
Clostridium botulinum	+	anearoob, (sporeforming)	+
Listeria monocytogenes	+	facultative	+ at 25°C , - at 37°C
Yersinia enterocolitica	-	facultative	+ at 25°C , - at 37°C

* Able to grow at temperatures <4°C

Between 1973 and 1987, bacterial infections accounted for 66% to 87% of all foodborne disease of known origin in the USA. In this 15-year period, several bacteria emerged that were not previously recognized as important foodborne pathogens, including *L. monocytogenes* and *Y. enterocolitica* [Bean & Griffin 1990]. Whilst these pathogens were increasingly involved in outbreaks of foodborne intoxications, refrigerated foods have, in many cases, been incriminated as the vehicles for these bacteria [Rocourt 1994; Bottone 1997; Kraft 1992]. Just like many mesophilic pathogens, these psychrotrophs appeared to be associated primarily with foods of animal origin. Extended refrigerated storage of raw and underprocessed foods of animal origin, as well as cross-contamination of other foods, have all played a role in outbreaks of both yersiniosis and listeriosis.

Cold-tolerant mesophiles

In addition to the expansion of these natural psychrotrophic species, recently some more or less cold-tolerant strains have also popped up in some typical mesophilic organisms, such as *Bacillus cereus*, *Escherichia coli* and *Salmonella* spp. [Palumbo 1986; d'Aoust 1991]. Such strains,

although not involved in human disease so far, may pose new problems for maintaining food safety.

2.4.3 Cold-tolerant Pathogens in Blood Products

Refrigeration has not been restricted to foods; amongst its numerous other applications, preservation of blood-products for transfusion is to be mentioned in view of it posing a risk to human health. Bacterial contamination of blood for transfusion is uncommon, but when such an event occurs, morbidity and mortality per individual may be significant [Mhyre 1985]. Among the micro-organisms involved in transfusion-associated bacteremia, *Y. enterocolitica* and *Pseudomonas fluorescens* have emerged to play an important role, undoubtedly favoured by their ability to grow at the common blood storage temperature of 4°C [Jacobs *et al*, 1981; Tipple *et al*. 1990].

2.5 FUTURE PERSPECTIVES

The present importance of refrigeration for the preservation of perishable foods can hardly be overestimated: the food supply to all our densely populated areas is nowadays, to a large extent, dependent upon chilling and freezing. This dependence is expected to be even further enhanced by ongoing urbanization, as the distance between production and consumption grows larger and larger [Persson 1987]. At the same time, foodborne disease is increasingly being recognized as a major cause of morbidity, and reduced economic productivity, in both industrialized and developing countries [Todd 1989; Archer 1985, Bean & Griffin 1990; Knöchel & Gould 1995] and cold-tolerant micro-organisms are jointly responsible for this.
Finally, cold-tolerant pathogens also pose a problem in modern medical technology, especially with regard to the safety of blood products.

REFERENCES

1. **Adams MR, and Moss MO.** 1995. *Food Microbiology*. The Royal Society of Chemistry, Cambridge, UK.
2. **Archer DL.** 1996. Preservation microbiology and safety: evidence that stress enhances virulence and triggers adaptation. *Trends Food Sci Technol* **7**: 91-95.
3. **Baumgartner E.** 1978. Grundsätzliches zur Haltbarmachung von Lebensmitteln. In: *Mikrobiologische Aspekte der Haltbarkeit von Lebensmitteln*, Schriftenreihe der Schweizerische Gesellschaft für Lebensmittelhygiene, heft 7. Vogt-Schild, Solothurn, Switzerland.
4. **Bean NH, and Griffin PM.** 1990. Foodborne disease outbreaks in the United States, 1973-1987: pathogens, vehicles, and trends. *J Food Prot* **53**: 804-817.
5. **Borgstrom G.** 1968. *Principles of food science*. The Macmillan Company, New York, USA, Vol I, pp 25-51; 106-107; 148-162.

6. **Bottone EJ.** 1997. *Yersinia enterocolitica*: the charisma continues. *Clin Microbiol Rev* **10**: 257-276.
7. **D'Aoust JY.** 1991. Psychrotrophy and foodborne *Salmonella*. *Int J Food Microbiol* **13**: 207-216.
8. **Fenton A, and Kisbán E (eds).** 1986. *Food in change; eating habits from the Middle ages to the present day*. John Donald Publishers, Edinburgh, UK.
9. **Forbes RJ.** 1966. *Studies in ancient technology*. EJ Brill, Leiden, the Netherlands. **Vol VI**, 2nd ed., pp 104-117.
10. **Gac A.** 1992. Les industries agro-alimentaires et la châine du froid. *Int J Refrig* **15**: 6-9.
11. **Gould GW** (ed.). 1989. *Mechanisms of action of food preservation procedures*. Elsevier applied science Publishers, London, UK, pp 1-10.
12. **Gould GW, Abee T, Granum PE, and Jones MV.** 1995. Physiology of food poisoning microorganisms and the major problems in food poisoning control. *Int J Food Microbiol* **18**: 121-128
13. **Gould GW.** 1996. Methods for preservation and extension of shelf life. *Int J Food Microbiol* **33**: 51-64.
14. **Hartog AP den.** 1980. De beginfase van het moderne voedselpatroon in Nederland. <in Dutch> (The early stages of modern eating patterns in the Netherlands) *Voeding* **41**: 334-341.
15. **Jacobs J, Jamaer D, Vandeven J, Wouters M, Vermylen C, Vandepitte J.** 1989. *Yersinia enterocolitica* in donor blood: a case report and review. *J Clin Microbiol* **27**: 1119-1121.
16. **Jobse-van Putten.** 1996. *Eenvoudig maar voedzaam. Cultuurgeschiedenis van de dagelijkse maaltijd in Nederland.* <in Dutch> (*Simple though substantial. The cultural history of the daily meal in the Netherlands*). SUN Memoria, Nijmegen, the Netherlands, pp 125-126, 451-452
17. **Jobse-van Putten J.** 1989. *Van pekelvat tot diepvrieskist.* <in Dutch> (*From the brine barrel to the deep freezer*). Van Meertens Instituut, Royal Dutch Society of Science (KNAW), Amsterdam, the Netherlands, pp 9-10.
18. **Knöchel S, and Gould G.** 1995. Preservation microbiology and safety: Quo Vadis? *Trends Foods Sci Technol* **6**: 127-131.
19. **Kraft AA.** 1992. *Psychrotrophic bacteria in foods; disease and spoilage.* CRC Press, Inc, Boca Raton, Florida, USA, pp 4-26, and pp 148-184
20. **Lechowich RV.** 1988. Microbial challenges of refrigerated foods. *Food Tech* **42**: 84-89.
21. **Legge J.** 1876. *The Chinese classics*, Vol III, The Shi King; or the book of poetry. Trübner, London, UK, pp 180-183.
22. **Leistner L.** 1995. Principles and applications of hurdle technology. In: *New methods of food preservation*. Gould GW (ed). Blackie Acadamic & Professional, London, UK, pp 1-21.
23. **Mekalanos JJ.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J Bacteriol* **174**: 1-7.
24. **Mez A.** 1922. *Die Renaissance des Islâms*. Heidelberg, Germany, pp 408-410.
25. **Mhyre BA.** 1985. Bacterial contamination is still a hazard of blood transfusion. *Arch Pathol Lab Med* **109**: 982-983.
26. **Mossel DAA, and Struijk CB.** 1991. Public health implication of refrigerated pasteurized ('sous-vide') foods. *Int J Food Microbiol* **13**: 187-206.
27. **Notermans S, Dufrenne J, and Lund BM.** 1990. Botulism risk of refrigerated, processed foods of extended durability. *J Food Prot* **53**: 1020-1024.
28. **Olsvik O, and Kapperud G.** 1982. Enterotoxin production in milk at 22 and 4 EC by *Escherichia coli* and *Yersinia enterocolitica*. *Appl Environ Microbiol* **43**: 997-1000.
29. **Otterlo A van.** 1990. *Eten en eetlust in Nederland 1840-1990.* <in Dutch> (*Food and appetite in The Netherlands* 1840-1990). Bert Bakker, Amsterdam, the Netherlands, pp 78-87 and p 175.
30. **Palumbo SA.** 1986. Is refrigeration enough to restrain foodborne pathogens? *J Food Prot* **49**: 1003-1009.
31. **Papworth JB.** 1832. (reprinted in 1971). *Rural residences.* Gregg International Publishers Ltd., Westmead, Farnborough, UK, p 98.
32. **Persson PO.** 1987. Refrigeration and its influence on the world food supply now and in the future. *Int J Refrig* **10**: 279-284.

33. **Plank R (ed).** 1954. *Handbuch der Kältetechnik*, band I: Entwicklung, wirtschaftliche Bedeutung, Wirkstoffe. Springer-Verlag, Berlin, Germany, pp 1-5, pp 97-117, and pp 146-150.
34. **Plank R (ed).** 1960. *Handbuch der Kältetechnik*, band X: Die Anwendung der Kälte in der Lebensmittelindustrie. Springer-Verlag, Berlin, Germany, p 1, and p 127.
35. **Plank R (ed).** 1962. *Handbuch der Kältetechnik*, band XI: Der gekühlter Raum, der Transport gekühlter Lebensmittel un die Eiserzeugung. Springer-Verlag, Berlin, Germany, pp 1-8, and pp 70-71.
36. **Potter NN, and Hotchkiss JH.** 1995. Cold preservation and processing. In: *Food Science.* Chapman & Hall, New York, USA, 5th ed, pp 163-199.
37. **Prescott SC, and Geer LP.** 1936. Observations on food poisoning organisms under refrigeration conditions. *Refrig Engineer* **32**:211-212, and 282-283.
38. **Reinink AW, and Vermeulen JG.** 1981. *IJskelders: koeltechnieken van weleer.* <in Dutch> (*Ice-houses: chilling techniques in olden times*). Heuff, Nieuwkoop, the Netherlands.
39. **Rocourt J.** 1994. *Listeria monocytogenes*: the state of the science. *Dairy, Food Environm San* **14**: 70-82.
40. **Sofos JN.** 1993. Current microbiological considerations in food preservation. *Int J Food Microbiol* **19**: 87-108.
41. **Sørhaug T, and Stepaniak L.** 1997. Psychrotrophs and their enzymes in milk and airy products: quality aspects. *Trends Food Sci Technol* **8**: 35-40.
42. **Tannahill R.** 1988. *Food in history*. Penguin Books Ltd, London, UK, pp 313-316.
43. **Thévenot R.** 1979. *A history of refrigeration throughout the world.* International Institute of Refrigeration, Paris, France.
44. **Tipple MA, Bland LA, Murphy JJ, Arduino MJ, Panlilio AL, et al.** 1990. Sepsis associated with transfusion of red cells contaminated with *Yersinia enterocolitica*. *Tranfusion* **30**: 207-213.
45. **Todd ECD.** 1989. Preliminary estimates of costs of foodborne disease in the United States. *J Food Prot* **52**: 595-601.
46. **Tompkin RB.** 1973. Refrigeration temperature as an environmental factor influencing the microbial quality of food. *Food Technol* **27**: 54-58.
47. **Toussaint-Samat M.** 1992. *History of food.* Blackwell, Oxford, UK, pp 749-754.
48. **Walker SJ, and Stringer MF.** 1990. Microbiology of chilled foods. In: *Chilled foods, the state of the art.* Gormley TR (ed). Elsevier Applied Science, London, UK, pp 269-304.

3

Bacteria in the Cold

adaptation & acclimation to low temperatures

ABSTRACT

Temperature has a profound influence on the metabolism and composition of most bacteria. Based on their upper and lower temperature limits of growth, four main groups can be distinguished, i.e. thermophiles, mesophiles, psychrotrophs and psychrophiles.

In response to a sudden decrease in growth temperature, many bacteria modify the fatty acid composition of their structural lipids. Although this modification is not a prerequisite for growth at low ambient temperatures, cold-adapted bacteria tend to have a higher degree of fatty acyl unsaturation in their membranes. In general, bacteria which are able to grow at temperatures near zero, the psychrophiles and psychrotrophs, seem to have developed the genetic ability to synthesize quantitatively or qualitatively more fluid lipids at low temperature than meso- or thermophiles, whose lower temperature limits for growth are around 7°C or 25°C, respectively. However, the relationship between the resulting phenotypic adaptations and the lower temperature limit for growth is still unclear.

The psychrophiles and psychrotrophs differ also from the meso- and thermophiles in their protein composition and -synthesis at reduced temperatures. Most bacteria, being either psychro-, meso- or thermophiles, produce a set of specific proteins, called 'cold-shock' proteins after an abrupt temperature down shift. According to the Cold-Shock Ribosomal Adaptation model, these cold-shock proteins enable them to adjust their mRNA translation machinery, especially their ribosomes, to reduced temperatures. The level of adjustment, however, seems to be species-specific. Meso- or thermophiles apparently fail to modify their ribosomes adequately to temperatures below 7°C or 25°C, respectively. Cold-adapted bacteria, on the other hand, are able to maintain efficient initiation of translation, even at temperatures near zero. During growth at such low temperatures, psychrophiles and psychrotrophs also synthesize another set of specific proteins, the so-called 'cold-acclimation' proteins, and these might be responsible for a far-reaching cold-adaptation of the translation machinery. Still, it is unclear to what extent these features contribute to setting the lower temperature limits for growth.

3.1 TEMPERATURE LIMITS FOR GROWTH

3.1.1 Restricted Growth Range

Bacteria as a group can grow over a wide temperature range that seems to be limited only by the availability of liquid water. Generally speaking this is between approximately -10 and +110°C. Individual species, however, show a much more limited growth range: their maximum and minimum temperatures are seldom more than 40 degrees apart (Figure 3.1).

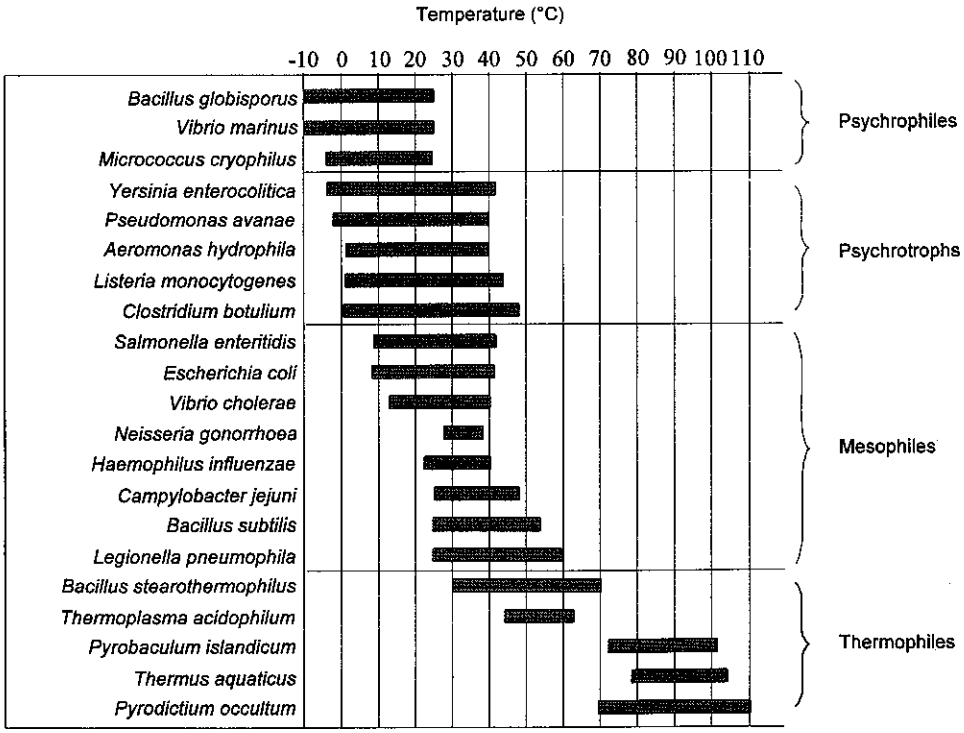


Figure 3.1 Growth temperature range of various bacteria, adapted from [Neidhardt *et al.* 1990]

According to their ability to grow at high, intermediate or low temperatures, micro-organisms have been divided into three broad classes: thermophiles, mesophiles and psychrophiles, respectively. The 'cold-loving' organisms, which can grow at temperatures close to zero, have been further subdivided into obligate and facultative psychrophiles, on the basis of their

optimum and upper growth temperatures [Morita 1975]. The optimum growth temperature of obligate psychrophiles lies below 16°C, whereas facultative psychrophiles, or psychrotrophs, grow best at around 20-25°C. Similarly, psychrotrophs have a higher upper temperature limit (up to 40°C) than psychrophiles (around 20°C).

3.1.2 Few Mutants with Dropped Lower Limits

Numerous studies have been carried out to determine why psychrophiles and psychrotrophs can grow at low temperature, or, from another point of view, why mesophiles and thermophiles cannot. To tackle this problem, there have been many attempts to isolate mutants from the group of mesophiles that have an extended lower temperature range. Such mutants, however, appeared to be extremely difficult to obtain. Despite numerous attempts, real psychrotrophic mutants of *E. coli* have never been isolated; the lowest minimum growth temperature obtained in some 'cold-resistant' mutants was 7.5°C [Kawamoto *et al.* 1989]. A mutant of the cyanobacterium *Anacystis nidulans* has been described, which had acquired enhanced tolerance to a temporary chilling stress but an extended growth range was not reported [Wada *et al.* 1994]. The only successful conversion of a mesophile into a psychrotroph reported so far concerns *Pseudomonas aeruginosa* [Azuma 1962; Olsen & Metcalf 1968].

The relative ease by which these mutants were obtained prompted the authors to suggest that the growth temperature range is prescribed by a limited number of genetic loci. However, this ease may only reflect the particular situation in *P. aeruginosa*: this organism probably requires a small number of mutations to gain psychrophily, since it is almost unique among Pseudomonads in not being itself a psychrotroph. The virtual inability to generate psychrotrophic mutants in other mesophilic species rather suggests that this would require mutations in a great number of genes.

3.1.3 Many Mutants with Raised Lower Limits

Reduced ability to grow near zero

In contrast to the apparent impracticability of a simultaneous 'upgrading' of all the key-genes for psychrotrophy in mesophiles, it is obviously less difficult to switch off one or more of these genes in psychrophiles.

'Cold-sensitive' mutants of psychrophiles, showing a reduced ability to grow at 0-10°C, have been frequently isolated. Such mutants appeared to be affected in various cellular processes. (Table 3.1, upper part). Growth-range-shifted mutants of *Micrococcus cryophilus* for example, which had swapped the ability to grow at 0°C for the ability to grow at elevated temperatures, showed changes in several metabolic pathways, such as oxidation and transamination routes [Tai & Jackson 1969]. On the other hand, no changes in metabolism, nor in ribosomal stability, were observed in certain mutants of *Bacillus psychrophilus*, which showed a similar shift in

growth range [Kim & Larkin 1973]. Some mutations seemed to have disordered membrane functioning, such as in a pure cold-sensitive mutant of *B. psychrophilus* [Murray & Innis 1980]: in this mutant, the ATPase synthesis is cold-inactivated and this might lead to an inability to energize membrane transport systems. In view of this, it is very interesting that various psychrotrophic bacteria of Antarctica, including *Pseudomonas, Micrococcus, Planococcus, Arthrobacter* and *Sphingobacterium* species, show temperature-dependent phosphorylation of membrane proteins and/or lipopolysacharides [Ray *et al.* 1994a, 1994b]. It was suggested from this observation that these membrane components might either function as sensors of external temperature or as modulators of membrane-permeability and transport activity. Membrane function is probably also affected in cold-sensitive mutants of the cyanobacterium *Synechocystis*, because their altered growth ranges coincide with dramatic changes in membrane lipid composition [Wada & Murata 1989]. More recently, three affected genes were isolated from cold-sensitive mutants of *Listeria monocytogenes*, but their products have not been identified [Zheng & Kathariou 1994, 1995].

Table 3.1 Affected cellular processes in various Cold-Sensitive mutants

AFFECTED CELLULAR PROCESSES	GENES	ORGANISMS	REFERENCES
oxidation and transamination	*?*	*Micrococcus cryophilus*	*110*
ATPase synthesis	*?*	*Bacillus psychrophilus*	*74*
membrane lipid desaturation	*desA*	*Synechocystis spec.*	*116 - 119*
Protein export	*secY*	*Escherichia coli*	*4*
DNA-precursor synthesis	*tmk*	*Escherichiacoli*	*25*
mRNA elongation	*nusA, nusB*	*Escherichia coli*	*18, 32*
Ribosome assembly	*?*	*Salmonella typhimurium*	*111*
Ribosome assembly	*rpsE*	*Escherichia coli*	*102*

'Cold-Sensitive' mesophiles

In addition to cold-sensitive mutants in psychrophilic species, mutants with raised lower temperature limits can also be found among meso- and thermophiles. Such mutants are generally also referred to as 'cold-sensitive' (CS), although their parent strains were never able to grow below 7°C. CS-mutants of *E. coli* and *Salmonella typhimurium*, for example, have lost the ability to grow below 20°C or even 30°C. Like the cold-sensitive mutants of psychrophiles, these CS-mutants also appear to be affected in diverse cellular processes, such as protein export, biosynthesis of DNA precursors, mRNA elongation, or ribosome assembly (Table 3.1, lower part).

3.1.4 Determinants of Minimum Growth Temperature

The coincidence of cold-sensitivity with the occurrence of disturbances in various biosynthesis-pathways suggests that adaptation to lower temperatures requires specific synthesis of macromolecules with particular characteristics. In addition, there are both physical [Russell 1990] and biochemical [Feller & Gerday 1997] reasons to suppose that the lower temperature limit for growth of a micro-organism is given by the sum of the characteristics of its macromolecules[1].

In other words, proteins and lipids are most likely the determinants of the minimum growth temperature. Indeed, numerous studies which were focused on the growth and physiology of micro-organisms have revealed that (i) temperature downshifts induce the production of specific macromolecules and (ii) there are quantitative and qualitative temperature-dependent differences in the cellular lipid- and protein compositions.

3.2 LIPID ADAPTATIONS

When micro-organisms are subjected to low temperatures, a variety of changes in lipid composition may occur, depending on the species involved [McElhaney 1976; Herbert 1986, 1989; Russell 1984a, 1984b, 1990, 1992; Gounot, 1991]. In response to cold, many micro-organisms, including *Y. enterocolitica* [Abbas &Card 1980; Nagamachi *et al.* 1991], accumulate lipids with a

1. **First**, the physics of aqueous solvent systems cannot explain why so many organisms are unable to grow at temperatures much above zero. Clearly, there is a physical restriction on growth at sub-zero temperatures: while between 0°C and -10°C many cells remain unfrozen, cytoplasmic waters begin to freeze when the temperature drops below -10°C. This results in toxic effects that will either prevent the organism from growing or possibly kill it. Thus, the minimum growth temperature of psychrotrophs and psychrophiles (-12°C) is fixed by the physical properties of aqueous solvent systems. The minimum growth temperatures of meso- (8°C) and thermophiles (25°C), however, is not given by such effects and must therefore reside in the chemical and/or structural properties of their cellular macromolecules. **Second**, thermodynamics also fails to explain why psychrophiles but not mesophiles can grow near zero. Certainly, temperature influences growth rates by the rate of enzyme reactions. Following the Arrhenius equation, which describes the exponential relationship between temperature and reaction rate, a drop in temperature of 10 degrees will usually produce a 2 to 3-fold decrease in enzyme rate constant. This means that a temperature shift from 37°C to 0°C will result in a 16 to 80-fold (2^4 to 3^4) lowering of enzyme activity. As a consequence, the velocity of all biochemical reactions may fall considerably when the temperature is lowered, but a definite standstill is not to be expected. Nevertheless, mesophilic and thermophilic enzymes usually completely lose their activity at lower temperatures, while psychrophilic proteins maintain their catalytic function. Apparently, cold-adapted bacteria have changed the properties of essential enzymes, whereas mesophiles have not. **Third**, when living organisms are subjected to temperature downshifts, many of them respond by implementing significant changes in their lipid- and protein compositions. This, too, indicates that adaptation to low temperature requires drastic quantitative and qualitative modifications in the macromolecules that are responsible for the cellular structure, organization and metabolism.

lower gel-to-liquid-crystalline transition temperature, thereby keeping membrane fluidity relatively constant [McElhaney 1976; McElhaney 1982; Herbert 1986; Cronan *et al.* 1987]. This phenomenon is called 'homeoviscous adaptation' [Russell 1984a; Herbert 1989][2].

3.2.1 Maintaining Membrane Fluidity

Homeoviscous adaptation has its origin in changes in the relative proportions of the various fatty acid classes present. It is brought about most commonly by an increase in the proportion of unsaturated residues and/or a decrease in the average fatty acyl chain length.

Increased fatty acid unsaturation

The major mechanism for regulation of the degree of unsaturation in *E. coli* is via anaerobic *de novo* synthesis of mono-unsaturated fatty acids [Mendoza & Cronan 1983; Cronan & Rock 1987]. Temperature dependent changes are mediated solely by the differential heat-sensitivity and relative activity of two enzymes with almost identical functions, but diverging precursor-affinities in the cyclic elongation pathway. The branching point between the synthesis of saturated and monounsaturated fatty acids is at the C10-phase, but the increase of unsaturation in *E. coli* at reduced temperature is completely brought about at the C16-phase, by an increased elongation of C16:1 (*palmitoleate*) compared to C16:0 (*palmitate*). This elongation step is catalyzed by either ß-ketoacyl-ACP synthase I or II, from which the latter enzyme does elongate C16:1 much more effectively than C16:0. In addition, synthase II is more temperature labile then synthase I. Consequently, the activity of the synthase II increases relative to that of the synthase I at reduced temperatures, and the amount of C18:1 (*cis-vaccenate*) will rise compared to its saturated counterpart C18:0 (*stearate*).

Reduced fatty acid chain length

The reduction of the average fatty acyl chain length is, in *E. coli*, also achieved by *de novo*

2. The classical phospholipid bilayer that forms the cytoplasmic and outer membranes of bacteria and other prokaryotes is not a rigid or unassailable barrier, but a fairly loosely packed, fluid and relatively permeable structure, which harbours embedded and traversing intrinsic proteins. When temperature falls, this flexible structure can undergo a reversible liquid-crystalline to gel phase transition, which blocks correct membrane function. However, important characteristics such as the efficiencies of electron transport, ion pumping and nutrient uptake (which have all often been mooted as determinants of psychrophily) depend, either directly or indirectly, on both membrane-lipid fluidity and -protein activity. For this reason, maintaining the liquid-crystalline state - and thus keeping membranes fluid and functional - is essential for microbial growth. Although quite wide variations in membrane fluidity are tolerated to sustain growth, micro-organisms have developed a number of strategies to ward off the imminent danger of membrane solidifying at low ambient temperatures. Membrane fluidity is profoundly affected by the fatty acid composition of the phospholipids: the shorter or less saturated the carbon chain is, the lower the 'melting point' of a fatty acid. Hence, the process of temperature-dependent membrane modification, termed 'homeoviscous adaptation' usually involves changes in the fatty acid moieties of the phospholipids.

synthesis of membrane phospholipids, and it is mediated by a temperature-regulated activity of the membrane-bound elongase enzyme. This enzyme can interconvert C16 and C18 fatty acids, and the conversion from C18:0 to C16:0 is maximal at low temperature.

Branched and cyclic fatty acids

Changes in the proportions or types of branched and cyclopropane fatty acids may also contribute to maintaining membrane fluidity, but are less frequently observed. Cyclopropane fatty acids are synthesized by addition of a methyl group across the cis-double bond of an unsaturated fatty acid acyl chain. Hence, C16:1 is the precursor of C17:0^{c}. In contrast to the synthesis of the straight-chain fatty acids, cyclopropane fatty acids are formed by modification of their precursors in the phospholipids, residing in the inner and outer membranes. In general, the amount of cyclopropane fatty acids rises when batch cultures enter stationary phase. However, at low pH, exponentially growing *Y. enterocolitica* cells were shown to synthesise rather large amounts of cyclic fatty acids, which was thought to increase the chance of survival under these conditions, by toughening the cellular membrane [Bodnaruk & Golden 1996]. The role of cyclopropane fatty acids in thermal adaptation, however, is questioned since their molecular packing properties are very similar to those of the monounsaturated precursors [Cronan & Rock,1987].

Combinations of fatty acid modifications

None of these different types of fatty acid modifications is unique for any class of micro-organism, either thermo-, meso- or psychrophilic, and all kinds of combinations are seen. These observations have led to the conclusion that a large variety of fatty acid compositions can apparently give the same thermal properties [Russell 1990].

3.2.2 High Unsaturation in Psychrotrophs and Psychrophiles

Despite the above conclusion, there is much evidence to show that the level of fatty acid unsaturation contributes to the lower temperature limit at which a bacterium can sustain growth. The mesophiles *E. coli* and *Arthrobacter globiformis*, for example, require a temperature-dependent minimum proportion of unsaturated fatty acids to sustain growth at reduced temperatures [Cronan & Gelman 1973; Canillac 1982]. However, the factual membrane lipid unsaturation levels are generally considerably in excess of the required minima [Herbert 1989; Russell 1990]. This might explain some rather confusing early observations that temporary starved, cold-shocked cells of *E. coli* resume growth at 10°C, while still having a fatty acid profile typical for cells grown at 37°C [Shaw & Ingraham 1965]. In fact, the fatty acid profiles of psychrotrophs and psychrophiles are often characterized by high degrees of unsaturation over their whole growth temperature range: e.g. >70% in certain *Pseudomonas* and *Vibrio* species, and even >95% in *Micrococcus cryophilus* [Russell 1984b, 1990; Herbert 1986]. The fatty acid profiles in these organisms are almost invariable with temperature, or only show a further

decrease in the, still low, average chain length. It has therefore been suggested that such high unsaturation levels provide a sufficiently fluid membrane throughout the whole growth temperature range, and chain length changes only represent a redundant control process or a 'fine-tuning' mechanism to regulate membrane-bound enzyme activity [Russell 1984b].

3.2.3 Specific Types of Unsaturation

In certain cyanobacteria, temperature affects not only the level but also the type of unsaturation: in *Synechocystis* species, temperature downshifts from 38°C to 22°C induce accumulation of tri- and tetraunsatured fatty acids at the expense of mono- and diunsaturated residues [Wada & Murata 1990]. Recently, it was shown that this shift in fatty acid profile is preceded by an increase in the mRNA levels of *desA, desB* and *desC* genes, which encode desaturases that introduce double bonds at particular positions in C18 fatty acids [Sakamoto *et al.* 1997]. Conversely, genetic manipulation of the extent and type of desaturation in *Synechocystis*, by means of mutations in one or more of the *des* genes, leads to aberrant growth patterns and shows that growth at low temperature requires either a certain level or a certain type of poly-unsaturation, or both [Wada & Murata 1989; Wada *et al.* 1992; Tasaka *et al.* 1996]. In addition, the tolerance of the photosynthetic machinery of *Synechocystis* to chilling stress was shown to depend on adaptational unsaturation of its membrane lipids [Gombos *et al.* 1994], and introduction of the cloned *desA* gene in the *desA*$^-$, chilling-sensitive cyanobacterium *Anacystis nidulans* increases the tolerance of the recipient to low temperature [Wada *et al.* 1990, 1994].

3.3 PROTEIN ADAPTATIONS

Temperature influences the protein composition of micro-organisms in three different ways: (i) proteins may undergo conformational changes, (ii) the rate of protein synthesis is down-regulated, or (iii) the protein synthesis is affected in a qualitative manner.

3.3.1 Changes in Protein Structure

At low temperatures, the rate of enzymatic reactions and the affinity of uptake- and transport systems decreases, while the requirement for organic substrates increases [Grauman & Marahiel 1996]. Thus, the structure of all the proteins in a cold-adapted micro-organism must be adjusted, such that conformational flexibility and catalytic efficiency are maintained for substrate conversions at low temperature. Obviously, this information must be fixed in the gene base-sequence of psychrophiles and psychrotrophs [Russell 1992]. A survey of the adaptive traits of various enzymes in psychrophilic organisms reveals that comformational flexibility, as well as catalytic efficiency, is certainly improved in comparison to their mesophilic

counterparts. This is mainly accomplished via the elimination of salt bridges and hydrophobic clusters, by a diminished interaction among aromatic side chains, or by an increase in the number of interactions between the enzyme surface and the solvent [Jaenicke 1990; Davail 1994; Marshall 1997]. At the molecular level, the determinants of the expected flexibility range from a single mutation in a functional site (but not the active one), to a large number of altered weak interactions, distributed in the whole structure [Feller & Gerday 1997].

3.3.2 Quantitative Downregulation of Protein Synthesis

When mesophiles like *E. coli* and *Pseudomonas aeruginosa* are subjected to temperatures that are below their minimal growth temperatures (± 8°C), their protein synthesis slows down progressively and eventually ceases [Das & Goldstein 1968]. This cessation of protein synthesis is accompanied by an accumulation of ribosomal subunits and 70S ribosomal particles, which are no longer capable of attaching to mRNA [Das & Goldstein 1968; Broeze *et al.* 1978]. Thus, these organisms are unable to grow at low temperatures due to a cold-induced block in initiation of translation. Such a block does not occur in the psychrotroph *Pseudomonas fluorescens* [Broeze *et al.* 1978] nor in a psychrophilic *Vibrio* species [Oshima *et al.* 1987], indicating that the translational capacity of their ribosomes is not affected by cold. This might be due to some positive conformational change of the ribosomes, or because a negative change fails to occur. Interestingly, a protein factor washed from a psychrotrophic *Pseudomonas putida* strain re-activated the translational capacity at 0°C of *E. coli* ribosomes [Szer 1970], suggesting a regulatory role for some initiation factor. Other studies revealed that cold-sensitivity does not reside in inactivation of initiation factors but is due to some feature of the ribosomal 30S subunit itself [Oshima *et al.* 1987]. Recently it was shown that the cold-sensitive phenotype of a certain *E. coli* mutant is caused by the absence of a protein that specifically interacts with 16S rRNA during 30S maturation [Dammel & Noller 1995].

The rate of protein turnover might also play a role in psychrotrophy, since it was observed that protein breakdown is much greater in the psychrotrophic bacterium *Arthrobacter* SI55 than in the mesophil *E. coli* [Potier *et al.* 1985]. Maybe, rapid protein turnover is an energy-saving mechanism to provide amino acids during the synthesis of new proteins, which are required for adaptation.

3.3.3 Qualitative Upregulation of Protein Synthesis

With respect to the intracellular protein composition, two types of cold-induced qualitative changes can be distinguished, i.e. 'cold-acclimation', which is observed during acclimatized growth at low temperature, and a 'cold-shock' response, which occurs after an abrupt temperature downshift.

Cold-acclimation-proteins: CAPs

Although the steady-state levels of most cellular proteins do not change greatly at different growth temperatures, some are synthesized at a greater level when a micro-organism is subjected to temperatures near the edge of its normal growth range. In *E. coli*, for example, three proteins are markedly increased during growth at 13.5°C, as compared with 37°C [Herendeen *et al.* 1979]. Similarly, many psychrophiles and psychrotrophs contain enhanced levels of certain proteins during continuous growth at low temperatures (≤ 7°C). This phenomenon is generally referred to as 'cold-acclimation', and the proteins of this specific class are called 'cold-acclimation' proteins or CAPs. CAPs have been detected in bacteria that belong to various genera, including *Aquaspirillum arcticum* [Roberts & Inniss 1992]; *Arthrobacter globiformis* [Potier *et al.* 1990] and *A. protophormiae* [Berger *et al.* 1996]; *Bacillus cereus* [Mayr *et al.* 1996], *B. psychrophilus* [Whyte & Inniss 1992] and *B. subtilis* [Graumann *et al.* 1996]; *Lactococcus lactis* [Panoff *et al.* 1994]; *Listeria monocytogenes* [Bayles *et al.* 1996]; *Pseudomonos fluorescens* [Ray *et al.* 1994c; Colucci & Innis 1996], *P. fragi* [Hébraud *et al.* 1994] and *P. putida* [Gumley & Innis 1996]; *Rhizobium* species [Cloutier *et al.* 1992]; and *Vibrio* species. [Araki 1991]. Recently, it was shown that CAPs are also produced (at 8°C) by *Enterococcus faecalis*, a bacterium that is generally described as a mesophil, although it is commonly present in cold environments [Panoff *et al.* 1997].

Cold-shock-proteins: CSPs

When bacteria are subjected to abrupt temperature down-shifts that do not exceed their normal growth range, their protein synthesis does not come to a halt, but is affected in a more subtle way: the production of house-keeping proteins is temporarily reduced in favour of a transient induction of a specific subset of proteins called 'cold-shock proteins' or CSPs. This specific pattern of gene-expression, which is generally referred to as the 'cold-shock-response', was first observed in the mesophile *E. coli* [Jones *et al.* 1987]. To date, the induction of cold-shock-proteins is known to occur in many other micro-organisms [Jones & Inouye 1994] and the phenomenon has been monitored in psychro-, meso-, and/or thermophilic species of the genera *Aquaspirillum* [Roberts & Inniss 1992], *Arthrobacter* [Ray *et al.* 1994c; Berger *et al.* 1996], *Bacillus* [Whyte & Inniss 1992; Willimsky *et al.* 1992; Lottering & Streips 1995; Graumann *et al.* 1996], *Enterococcus* [Panoff *et al.* 1997], *Lactobacillus* [Mayo *et al.* 1997], *Lactococcus* [Panoff *et al.* 1994], *Listeria* [Phan-Thanh & Gormon 1995; Bayles *et al.* 1996], *Pseudomonas* [Ray *et al.* 1994c; Colucci & Innis 1996; Gumley & Innis 1996], *Rhizobium* [Cloutier *et al.* 1992], *Salmonella* [Jeffreys *et al.* 1998] and *Vibrio* [Araki 1991]. Very recently, a cold shock response has also been reported for *Yersina enterocolitica* [Dickinson *et al.* 1998] and *Y. pseudotuberculosis* [Tafelshtein *et al.* 1998].

Most of the CSPs produced by *E. coli*, as well as the cellular processes in which they are involved, have now been identified (Table 3.2).

Table 3.2 The Cold Shock response in *E.coli* :
Cold shock proteins, their corresponding genes, and the (supposed) affected cellular processes

COLD SHOCK PROTEINS	GENES	AFFECTED CELLULAR PROCESSES	REF.
CspA (or CS7.4)	*cspA*	Transcription initiation Transcription	*56*
NusA	*nusA*	termination	*56*
Initiation factor 2α, β	*infB*	Translation initiation	*54*
Ribosomal binding factor A (P15)	*rbfA*	Ribosomal maturation,	*54*
CsdA (or DeaD)	*csdA*	mRNA unwinding,	*59*
Polynucleotide phosphorylase	*pnp*	mRNA degradation	*56*
Pyruvate dihydrogenase	*aceE,F*	Pyruvate-decarboxylation	*56*
RecA	*recA*	DNA repair	*56*
H-NS	*hns*	DNA structuring	*64*
DNA gyrase α-subunit	*gyrA*	DNA structuring	*58*
Trigger factor	?	Ribosomal modification & Polypeptide folding	*47*

The *E. coli* cold-shock protein with the highest induction level (200-fold increase following a shift from 37°C to 10°C) is CspA (or CS7.4). The induction of most other *E. coli* CSPs is thought to be under transcriptional control of CspA, and the transcription enhancement of the cold-shock genes is most likely mediated by a specific recognition site in, or nearby, their promoters [LaTeana 1991; Jones *et al.* 1992b; Qoronfleh *et al.* 1992; Jones & Inouye 1994; Brandi *et al.* 1994]. This recognition site comprises the 5-base motif ATTGG, or its inverted repeat CCAAT, and is identical to the so-called 'Y-box', the recognition site for eukaryotic homologs of CspA [Wolffe 1994; Jones *et al.* 1992b]. These motifs are now generally referred to as 'cold-shock motifs'. In addition, CspA shares the binding capacity to the cold-shock motif with a whole family of homologs in *E. coli*, three of which, CspB, G and I, are also cold-inducible [Lee *et al.* 1994; Nakashima *et al.* 1996; Wang *et al.* 1999].

Overlap CAPs and CSPs

Comparative studies have shown that there is considerable, but not complete, overlap between the proteins that are specific for cold-acclimation and the cold-shock-response. This overlap is seen in several cold-adapted organisms, including *Aquaspirillum arcticum* [Roberts & Inniss 1992]; *Bacillus psychrophilus* [Whyte & Inniss 1992]; *Pseudomonas fluorescens* [Ray *et al.* 1994c] and *P. putida* [Gumley & Inniss 1996]; *Enterococcus faecalis* [Panoff *et al.* 1997] and *Vibrio* spec. [Araki 1991]. Interestingly, CAPs which are structurally highly homologous to the major *E. coli* cold-shock-protein CspA have been found in various, phylogenetically unrelated micro-organisms (Table 3.3).

Table 3.3 CAPs and CSPs which are homologs of the major *E. coli* cold shock protein CspA, in various psychrotrophic (❄) or mesophilic bacteria.

ORGANISMS	COLD ADAPTATION PROTEINS	COLD SHOCK PROTEINS	REFERENCES
Arthrobacter globiformis ❄	CspA-like	CapA	*7*
Bacillus cereus ❄	CspA-like	---*	*69*
Escherichia coli	---	CspB, CspG, CspI	*65, 76, 121*
Lactococcus lactis ❄	---	CspB	*15, 63*
Lactobacillus plantarum	---	CspL, CspP	*68*
Listeria monocytogenes ❄	CspA-like	---	*6*
Bacillus subtilis	---	CspB, CspC, CspD	*38, 39, 123*
Pseudomonas fluorescens ❄	CspA-like	---	*93*
Pseudomonas fragi ❄	---	C7.0, C8.0	*43*
Salmonella enteritidis	---	CspA	*49*
Yersinia enterocolitica ❄	---	CspB	*27*

* --- = not investigated, or not detected

Extracellular anti-freeze proteins

Some bacteria are reported to produce extracellular anti-freeze proteins. Such proteins are synthesized and secreted by numerous species of fish, insects and plants, especially those from cold habitats, to protect their body-tissues from freezing/thawing damage. This protection is accomplished by the control of extracellular ice-formation in their body-fluids, through a process called thermal hysteresis. With bacteria, the thermal hysteresis activity of anti-freeze proteins might likewise function to regulate the growth of ice-crystals outside the bacterium, thereby protecting it from freezing-damage. Thermal hysteresis activity has been observed in extracts from the common soil bacterium *Rhodococcus erythropolis* and the psychrotroph *Micrococcus cryophilus* [Duman & Olsen 1993], and an anti-freeze protein has been isolated from the rhizobacterium *Pseudomonas putida* [Sun *et al.* 1995]. However, the thermal hysteresis activity was not observed until after 3 to 4 weeks of cold growth. This suggests that the anti-freeze proteins are not linked with the processes which enable these bacteria to function at low temperatures.

3.4 REGULATION OF COLD-ADAPTATION

3.4.1 Cold Shock Proteins: Effectors of Cold-Adaptation?

Several lines of evidence suggest that the cold-shock response is essential for bacterial

adaptation to low temperature. **First**, the lag period of cell growth upon cold shock corresponds to the period of transient expression of the cold-shock genes [Jones *et al.* 1992a]. **Second**, there is a reciprocal relationship between the production of cold-shock proteins and the production of other cellular proteins [Jiang *et al.* 1996a]. **Third**, the absence of certain cold-shock proteins frequently leads to reduced viability, especially at low temperatures. *E. coli* mutants with a truncated *hns* gene, for example (which are devoid of a functional DNA-binding protein H-NS), proliferate more slowly than the wild type at 12°C, but not at 37°C [Dersch *et al.* 1994]. Similarly, *rbfA* knock out mutants, which lack the ribosomal binding factor RbfA, can't grow at 26°C and below [Dammel & Noller 1995]. Likewise, the intracellular amounts of the initiation factors IF2α and IF2ß, encoded by *infB*, seem to be crucial at reduced temperatures, since *E. coli* mutants lacking one of these forms can't grow at 30°C and below [Sacerdot *et al.* 1992]. In addition, a minimum of one *csp* gene (B, C or D) is essential for viability of *B. subtilis*, although this effect seems not to be restricted to low temperatures [Graumann *et al.* 1997]. **Fourth**, over-expression of cold-shock genes also has profound effects on viability: plasmid-mediated over-production of RbfA results in faster resumption of growth after a temperature downshift [Jones & Inouye 1996]. Overproduction of truncated *cspA* mRNAs (which retain translational ability), on the other hand, completely blocks cell growth at low temperatures [Jiang *et al.* 1996b].

3.4.2 Ribosomes: Sensors for Cold-Adaptation?

On the basis of various observations with respect to ribosomal assembly, functioning and maturation [Das 1968; Nashimoto & Nomura 1970; Broeze *et al* 1978; Har-El *et al.*1979], a sensor function was postulated for the ribosome in various bacterial stress-responses, including the heat- and cold-shock responses [VanBogelen & Neidhardt 1990]. This idea was supported by more recent studies, in which was shown that (i) several cold-sensitive mutants of *E. coli* are defective in proper assemblage of ribosomes [Yano & Yura 1989]; (ii) various cold-shock proteins, such as RbfA, CsdA and IF2, have functions associated with the ribosome [Dammel & Noller 1995; Jones *et al.* 1996]; and (iii) antibiotics which target the prokaryotic ribosome, such as chloramphenicol, tetracycline and erythromycin can mimic temperature shifts, including the corresponding induction of heat- or cold-shock proteins [Jiang et al. 1993; VanBogelen *et al.* 1990]. Moreover, various 'cold'-sensitive mutants of *E. coli* have been described that were affected in either transcription termination, translation initiation, or polypeptide elongation at 20°C or below [Nashimoto *et al.* 1985; Shiba et al.1986a; Shiba et al.1986b; Schauer et al.1987]. Finally, the mRNA of *cspA* is very unstable at 37°C, but is transiently stabilized at 15°C [Tanabe *et al.* 1992; Goldenberg *et al.* 1996]. Thus, several pieces of evidence suggest that inhibition of translation is setting the minimal temperature of bacterial growth, and the adaptive role of the cold-shock response is in the adequate modification of the translation machinery.

3.4.3 The 'Cold Shock Ribosomal Adaptation' model

To explain the induction and function of the cold-shock -response, the 'cold-Shock Ribosomal Adaptation model' was proposed [Jones & Inouye 1996] (Figure 3.2).

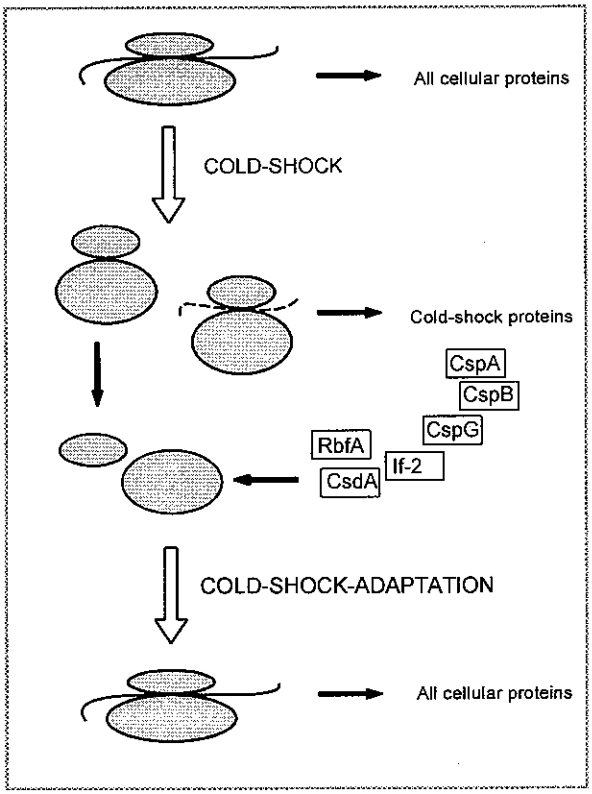


Figure 3.2 The Cold Shock Ribosomal Adaptation model, adapted from [Jiang *et al.* 1996b]

In this model, ribosomes are translatable at high and moderate temperatures, but become defective in initiation of translation of most mRNAs after a temperature downshift. The mRNA of *CspA* and other cold-shock genes, by contrast, can still be efficiently translated. Thus, the cold-induced change in translational efficiency of the ribosomes triggers the synthesis of CspA, which snowballs into en-hanced transcription and synthesis of Initiation Factor 2, RbfA, and CsdA. These CSPs associate with 70S monosomes and with 30S or 50S ribosomal subunits. Through this interaction, the ribosomes are converted to a renewed translatable, cold-resistant state.

By this, adaptation to the lower temperature has been accomplished: the synthesis of housekeeping proteins will resume, and the cell can start growing again.

3.4.4 Onset and Shutdown of the Cold Shock Response

The likelihood of the above scenario is supported by several new observations with respect to mechanisms of onset and shutdown of the cold-shock response. It was shown, for example, that cold-shocked ribosomes translate the *cspA* mRNA much more efficiently compared to ribosomes from 37°C-grown cells [Brandi *et al.* 1996]. Furthermore, the cold-shock induction of

CspA appeared to be promoter-independent, i.e. the *cspA* gene is efficiently transcribed even at 37°C, but the translation of the *cspA* mRNA is blocked because of its extreme instability at 37°C [Fang *et al.* 1997]. Moreover, a possible explanation was found for the temperature-independent maintenance of the ribosome's translation efficiency with respect to the *cspA* mRNA's: the cold-shock induction of CspA required the presence of a certain 14-base stretch in the 5'-terminus of the coding region [Mitta *et al.* 1997]. This stretch, which is called the Downstream Box, is complementary to a 16S rRNA region, and was previously shown to act as an efficient translation initiation signal in *E. coli* [Sprengart *et al.* 1996]. It now appeared to be highly conserved in the m-RNA of all cold shock proteins. Comparably, another conserved sequence is supposed to play a role in the ending of the cold-shock response. This concerns an 11-base motif in the 5' untranslated region (5'-UTR) of the many CSP's mRNA's [Jiang *et al.* 1996a]. This motif is designated the 'cold box', and it may act as a repressor binding site [Jiang *et al.* 1996b; Yamanaka *et al.*1998; Fang *et al.* 1998].

3.4.5 Cold Shock Proteins: mRNA-chaperones?

In addition to the major problems that result from a temperature fall, i.e. the reduction of membrane fluidity and the impaired protein synthesis, cold-shocked cells are also confronted with an increase in the stability of secondary structures in DNA and mRNA [Thieringer *et al.* 1998]. Stable secondary structures affect the efficiencies of transcription, translation and DNA-replication. CSPs are, for several reasons, now thought to play a role in counteracting these effects. First, CSPs prevent formation of secondary structures within the 5'-end of mRNA [Graumann *et al.*1997]; second, CsdA was shown to be exclusively associated with ribosomes and to exhibit dsRNA unwinding activity [Jones *et al.* 1996]; and third, CspA was shown to bind co-operatively to ssRNA and ssDNA, thus protecting it from denaturation [Jiang *et al.* 1997]. Furthermore, PNPase, which is one of the two 3'-5' exoribonucleases and, at the same time, one of the minor cold shock proteins in *E. coli*, can degrade secondary-structured mRNAs more efficiently than its counterpart RNaseII [Guarneros & Portier 1990]. Moreover, PNPase was found to act in concert with several other enzymes, including the endonuclease RNaseE, which is involved in the general degradation of the bulk of cellular mRNAs, and the RNA-helicase RhlB [Carpousis *et al.*1994; Braun *et al.* 1996; Pye *et al.* 1996]. This multi-enzyme complex, which was designated 'degradosome', is thought to co-ordinate two processes, i.e. the decay of intermediates that would encode non-functional polypeptides on the one hand, and the efficient recycling of ribonucleotides, on the other [Pye *et al.* 1994]. Recently, another cold-shock protein was detected, called Trigger Factor, that appeared to be associated with ribosomes and nascent polypeptides [Hesterkamp *et al.*1997]. This protein is also thought to be a molecular chaperone, probably engaged in proper folding at low temperature [Kandror & Goldberg 1997].

3.4.6 Insufficient Cold Shock Response in Mesophiles?

The striking omnipresence of CspA-like proteins in cold-adapted micro-organisms has lead to the idea that proteins of this family might play a role in protecting cells from damage due to cold [Goldstein *et al.* 1990]. In line with this, it has been speculated that mesophilic bacteria fail to restore harmonious growth at low temperatures because their cold-shock response is probably only a part of the total response of psychrotrophs and psychrophiles [Berger *et al.* 1996]. This hypothesis is supported by the results of comparative studies addressing the CSP-profiles of wild-type and mutant strains of *Bacillus psychrophilus*, since two CSPs of the parental strain are not induced in a cold-sensitive mutant [Whyte & Inniss 1992]. On the other hand, studies on the CSP-profile of *Listeria monocytogenes* showed no obvious differences between the wild type and a cold-sensitive mutant [Bayles *et al.* 1996]. Introduction of CSP- or CAP-genes from psychrotrophs into mesophiles might shed light on the physiological role of the cold-shock-response and the cold-acclimation proteins, but such experiments have not been described so far.

REFERENCES

1. **Abbas CA, and Card GL.** 1980. The relationships between growth temperature, fatty acid composition and the physical state and fluidity of membrane lipids in *Yersinia enterocolitica*. *Biochim Biophys Acta* **602**: 469-476.
2. **Araki T.** 1991. The effect of temperature shifts on protein synthesis by the psychrophilic bacterium *Vibrio* sp. strain ANT-300. *J Gen Microbiol* **137**: 817-826.
3. **Azuma Y, Newton SB, and Witter LD.** 1962. Production of psychrophilic mutants from mesophilic bacteria by ultraviolet irradiation. *J Dairy Sci* **45**: 1529-1530.
4. **Baba T, Jacq A, Brickman E, Beckwith J, Taura T, Ueguchi C, Akiyama Y, and Ito K.** 1990. Characterization of cold-sensitive *secY* mutants of *Escherichia coli*. *J Bacteriol* **172**: 7005-7010.
5. **Bayles DO, Bassam AA, and Wilkinson BJ.** 1996. Cold stress proteins induced in *Listeria monocytogenes* in response to temperature downshock and growth at low temperatures. *Appl Environ Microbiol* **62**: 1116-1119.
6. **Berger F, Normand P, and Potier P.** 1997. *CapA*, a *CspA*-like gene that encodes a cold acclimation protein in the psychrotrophic bacterium *Arthrobacter globiformis* SI55. *J Bacteriol* **179**: 5670-5676.
7. **Berger F, Morellet N, Menu F, and Potier P.** 1996. Cold shock and cold acclimation proteins in the psychrotrophic bacterium *Arthrobacter globiformis* SI55. *J Bacteriol* **178**: 2999-3007.
8. **Bodnaruk PW, Golden DA.** 1996. Influence of pH and incubation temperature on fatty acid composition and virulence factors of *Yersinia enterocolitica*. *Food Microbiol 13: 17-22.*
9. **Brandi A, Pon CL, and Gualerzi CO.** 1994. Interaction of the main cold-shock protein CS7.4 (CspA) of *Escherichia coli* with the promoter region of *hns*. *Biochimie* **76**: 1090-1098.
10. **Brandi A, Pietroni P, Gualerzi CO, and Pon CL.** 1996. Post-transcriptional regulation of CspA expression in *Escherichia coli*. *Mol Microbiol* **19**: 231-240.
11. **Braun F, Hajnsdorf E, and Régnier P.** 1996. Polynucleotide phosphorylase is required for the rapid degradation of the RnaseE processed *rpsO* mRNA of *Escherichia coli* devoid of its 3' hairpin. *Mol Microbiol* **19**: 997-1005.
12. **Broeze RJ, Carol JS, and Pope DH.** 1978. Effects of low temperature on in vivo and in vitro protein synthesis in *Escherichia coli* and *Pseudomonas fluorescens*. *J Bacteriol* **134**: 861-874.

13. **Canillac N.** 1982. Effet de la température d'incubation sur la composition lipidique de Corynébacteriacées du genre *Arthrobacter*. *Can J Microbiol* **28**: 284-290.
14. **Carpousis AJ, Van Houwe G, Ehretsmann C, and Krisch HM.** Copurification of *E. coli* RNaseE and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation. *Cell* **76**: 889-900.
15. **Chapot-Chartier MP, Schouler C, Lepeuple AS, Gripon JC, and Chopin MC.** 1997. Characterization of *cspB*, a coldshock-inducible gene from *Lactococcus lactis*, and evidence for a family of genes homologous to the *Escherichia coli cspA* major cold shock gene. *J Bacteriol* **179**: 5589-5593.
16. **Cloutier J, Prévost D, Nadeau P, and Antoun H.** 1992. Heat and cold shock protein synthesis in Arctic and temperate strains of Rhizobia. *Appl Environ Microbiol* **58**: 2846-2853.
17. **Colucci M, and Inniss WE.** 1996. Ethylene glycol utilization, cold and ethylene glycol shock and acclimation proteins in a psychrotrophic bacterium. *Curr Microbiol* **32**: 179-182.
18. **Craven MG, and Friedman DI.** 1991. Analysis of the *Escherichia coli nusA10*(Cs) allele: relating nucleotide changes to phenotypes. *J Bacteriol* **173**: 1485-1491.
19. **Cronan JE, and Gelman EP.** 1973. An estimate of the minimum amount of unsaturated fatty acids required for growth of *Escherichia coli*. *J Biol Chem* **248**: 1188-1195.
20. **Cronan Jr. JE, Gennis RB, and Maloy SR.** 1987. Cytoplasmic membrane. In: *Escherichia coli and Salmonella typhimurium. Cellular and molecular biology*. Neidhardt FC (ed). American Society for Microbiology,Washington DC, USA, pp 31-55.
21. **Cronan JE Jr., and Rock CO.** 1987. Biosynthesis of membrane lipids. *Escherichia coli* and *Salmonella typhimurium*. Neidhardt FC (Ed). American Society for Microbiology,Washington DC, USA, Vol 1, pp 474-497.
22. **Dammel CS, and Noller HF.** 1995. Suppression of a cold-sensitive mutation in 16S rRNA by overexpression of a novel ribosome-binding factor, RbfA. *Genes Dev* **9**: 626-637.
23. **Das HK, and Goldstein A.** 1968. Limited capacity for protein synthesis at zero degrees centigrade in *Escherichia coli*. *J Mol Biol* **31**: 209-226.
24. **Davail S, Feller G, Narinx E, and Gerday C.** 1994. Cold adaptation of proteins. *J Biol Chem* **269**: 17448-17453.
25. **Daws TD, and Fuchs JA.** 1984. Isolation and characterization of an *Escherichia coli* mutant deficient in dTMP kinase activity. *J Bacteriol* **157**: 440-444.
26. **Dersch P, Kneip S, and Bremer E.** 1994. The nucleoid-associated DNA-binding protein H-NS is required for the efficient adaptation of *Escherichia coli* K-12 to a cold environment. *Mol Gen Genet* **245**: 255-259.
27. **Dickinson JH, Francis KP, Throup JP and Stewart GSAB.** 1998. The cold shock response of *Yersinia enterocolitica*. Oral presentation at the 7th International Congress on Yersinia, Nijmegen, The Netherlands. *Ned Tijdsch Med Microbiol* Supp II **6**: 8.
28. **Duman JG, and Olsen TM.** 1993. Thermal hysteresis protein activity in bacteria, fungi and fylogenetically diverse plants. *Cryobiol* **30**: 322-328.
29. **Fang L, Hou Y, and Inouye M.** 1998. Role of the cold-box region in the 5'-untranslated region of cspA mRNA in its transient expression at low temperature in *Escherichia coli*. *J Baxcteriol* **180**: 90-95
30. **Fang L, Jiang W, Bae W, Inouye M.** 1997. Promoter-independent cold-shock induction of *cspA* and its derepression at 37°C by mRNA stabilization. *Mol Microbiol* **23**: 355-364.
31. **Feller G, and Gerday C.** 1997. Psychrophilic enzymes: molecular basis of cold adaptation. *Cell Mol Life Sci* **53**: 830-841.
32. **Georgopoulos CP, Swindle J, Keppel F, Ballivet M, Bisig R, and Eisen H.** 1980. Studies on the *E. coli groNB* (*nusB*) gene which affects bacteriophage lambda N gene function. *Mol Gen Genet* **179**: 55-61.
33. **Goldenberg D, Azar I, and Oppenheim AB.** 1996. Differential mRNA stability of the CspA gene in the cold-shock response of *Escherichia coli*. *Mol Microbiol* **19**: 241-248.
34. **Goldstein J, Pollitt NS, and Inouye M.** 1990. Major cold shock protein of *Escherichia coli*. *Proc Natl Acad Sci* USA **87**: 283-287.

35. **Gombos Z, Wada H, and Murata N.** 1994. The recovery of photosynthesis from low-temperature photoinhibition is accelerated by the unsaturation of membrane lipids: a mechanism of chilling tolerance. *Proc Natl Acad Sci* USA **91**: 8787-8791.
36. **Gounot AM.** 1991. Bacterial life at low temperature: physiological aspects and biotechnological implications. *J Appl Bacteriol* **71**: 386-397.
37. **Graumann P, and Marahiel MA.** 1996. Some like it cold: response of micro-organisms to cold shock. *Arch Microbiol* **166**: 293-300.
38. **Graumann P, Schröder K, Schmid R, and Marahiel MA.** 1996. Cold shock stress-induced proteins in *Bacillus subtilis*. *J Bacteriol* **178**: 4611-4619.
39. **Graumann P, Wendrich TM, Weber MHW, Schröder K, and Marahiel MA.** 1997. A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. *Mol Microbiol* **25**: 741-756.
40. **GuarnerosG, and Portier C.** 1990. Different specificities of ribonuclease II and polynucleotide phosphorylase in 3'-mRNA decay. *Biochimie* **72**: 771-777.
41. **Gumley AW, and Inniss WE. 1996.** Cold shock proteins and cold acclimation proteins in the psychrotrophic bacterium *Pseudomonas putida* Q5 and its transconjugant. *Can J Microbiol* **42**: 798-803.
42. **Har-El R, Silberstein A, Kuhn J, Tal M.** 1979. Synthesis and degradation of *lac* mRNA in *E. coli* depleted of 30S ribosomal subunits. *Mol Gen Genet* **173**: 135-144.
43. **Hébraud M, Dubois E, Potier P, and Labadie J.** 1994. Effect of growth temperatures on the protein levels in a psychrotrophic bacterium, *Pseudomonas fragi*. *J Bacteriol* **176**: 4017-4024.
44. **Herbert RA.** 1986. The ecology and physiology of psychrophilic micro-organisms. In: *Microbes in extreme environments*. Herbert RA, and Codd GA (eds). Academic Press, London, UK, pp 1-23.
45. **Herbert RA.** 1989. Microbial growth at low temperature. In: *Mechanism of action of food preservation procedures*. Gould GW (ed). Elseviers Science Publishers, London, UK, pp 71-96.
46. **Herendeen SL, VanBogelen RA, and Neidhardt FC.** 1979. Levels of major proteins of *Escherichia coli* during growth at different temperatures. *J Bacteriol* **139**: 185-194.
47. **HesterkampT, Deuerling E, and Bukau B.** 1997. The amino-terminal 118 amino acids of *Escherichia coli* trigger factor consitute a domain that is necessary and sufficient for binding to ribosomes. *J Biol Chem* **272**: 21865-21871.
48. **Jaenicke R.** 1990. Protein structure and function at low temperatures. *Phil Trans R Soc Lond B* **326**: 535-553.
49. **Jeffreys AG, Hak KM, Steffan RJ, Foster JW, and Bej AK.** 1998. Growth, survival and characterization of *CspA* in *Salmonella enteritidis* following cold shock. *Curr Microbiol* **36**: 29-35.
50. **Jiang W, Jones P, Inouye M.** 1993. Chloramphenicol induces transcrioption of the major cold shock gene of *Escherichia coli*: *cspA*. *J Bacteriol* **175**: 5824-5828.
51. **Jiang W, Fang L, and Inouye M.** 1996a. The role of the 5'-end untranslated region of the mRNA for CspA, the major cold-shock protein of *Escherichia coli*, in cold-shock adaptation. *J Bacteriol* **178**: 4919-4925.
52. **Jiang W, Fang L, and Inouye M.** 1996b. Complete growth inhibition of *Escherichia coli* by ribosome trapping with truncated *cspA* mRNA at low temperature. *Genes to Cells* **1**: 965-976.
53. **Jiang W, Hou Y, and Inouye M.** 1997. CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. *J Biol Chem* **272**: 196-202.
54. **Jones PG, and Inouye M.** 1994. The cold-shock response - a hot topic. *Mol Microbiol* **11**: 811-818.
55. **Jones PG, and Inouye M.** 1996. RbfA, a 30S ribosomal binding factor, is a cold-shock protein whose absence triggers the cold shock response. *Mol Microbiol* **21**: 1207-1218.
56. **Jones PG, VanBogelen RA, and Neidhardt FC.** 1987. Induction of proteins in response to low temperature in *Escherichia coli*. *J Bacteriol* **169**: 2092-2095.
57. **Jones PG, Cashel M, Glaser G, and Neidhardt FC.** 1992a. Function of a relaxed state following temperature downshifts in *Escherichia coli*. *J Bacteriol* **174**: 3903-3914.
58. **Jones PG, Krah R, Tafuri SR, and Wolffe AP.** 1992b. DNA gyrase, CS7.4 and the cold shock

response in *Escherichia coli*. *J Bacteriol* **174**: 5798-5802.
59. **Jones PG, Mitta M, Kim Y, Jiang W, and Inouye M.** 1996. Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. *Proc Natl Acad Sci* USA **93**: 76-80.
60. **Kandror O, and Goldberg AL.** 1997. Trigger factor is induced upon cold shock and enhances viability of *Escherichia coli* at low temperatures. *Proc Natl Acad Sci USA* **94**: 4978-4981.
61. **Kawamoto S, Yasokawa D, Kaisou A, Tokuyama S, Aoyama K, Sasabuchi M, and Yashima S.** 1989. Characterization of a cold-resistant mutant of *Escherichia coli*. *J Ferm Bioengin* **68**: 383-389.
62. **Kim KJ, and Larkin JM.** 1973. Production of mesophilic mutants from a psychrophilic *Bacillus*. *Can J Microbiol* **19**: 1452-1454.
63. **Kim WS, and Dunn NW.** 1997. Identification of a cold shock gene in Lactic Acid Bacteria and the effect of cold shock on cryotolerance. *Curr Microbiol* **35**: 59-63.
64. **LaTeana A, Brandi A, Falconi M, Spurio R, Pon CL, and Gualerzi CO.** 1991. Identification of a cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleoid protein H-NS. *Proc Natl Acad Sci* USA **88**: 10907-10911.
65. **Lee SJ, Xie A, Jiang W, Etchegaray JP, Jones PG, and Inouye M.** 1994. Family of the major cold-shock protein, CspA (CS7.4), of *Escherichia coli*, whose members show a high sequence similarity with the eukaryotic Y-box binding proteins. *Mol Microbiol* **11**: 833-839.
66. **Lottering EA, and Streips UN. 1995.** Induction of cold shock proteins in *Bacillus subtilis*. *Curr Microbiol* **30**: 193-199.
67. **Marshall CJ.** 1997. Cold-adapted enzymes. *Tibtech* **15**: 359-364.
68. **Mayo B, Derzell S, Fernández M, Léonard C, Ferain T, Hols P, Suárez JE, and Delcour J.** 1997. Cloning and characterization of *cspL* and *cspP*, two cold-inducible genes from *Lactobacillus plantarum*. *J Bacteriol* **179**: 3039-3042.
69. **Mayr B, Kaplan Th, Lechner S, and Scherer S.** 1996. Identification and purification of a family of dimeric major cold shock protein homologs from the psychrotrophic *Bacillus cereus* WSBC 10201. *J Bacteriol* **178**: 2916-2925.
70. **McElhaney RN.** 1976. The biological significance of alterations in the fatty acid composition of microbial membrane lipids in response to changes in the environmental temperature. In:*Extreme environments: mechanisms of microbial adaptation*. Heinrich MR (ed). Academic Press,New York, USA, pp. 255-281.
71. **Mendoza E de, and Cronan JE Jr.** (1983). Thermal regulation of membrane lipid fluidity in bacteria. *Trends Biochem Sci* **8**, 49-52
72. **Mitta M, Fang L, and Inouye M.** 1997. Deletion analysis of cspA of Escherichia coli: requirementof the AT-rich UP element for *csp*A transcription and the downstream box in the coding region for its cold shock induction. *Mol Microbiol* **26**: 321-335.
73. **Morita RY.** 1975. Psychrophilic bacteria. *Bacteriol Rev* **39**: 146-147.
74. **Murray WD, and Inniss WE.** 1980. Cold-sensitive mutant with defective growth at 5EC from a psychrotrophic bacterium. *Can J Microbiol* **26**: 1-6.
75. **Nagamachi E, Shibuya S, Hirai Y, Matsushita O, Tomochika K, and Kanemasa Y.** 1991. Adaptional changes of fatty acid composition and the physical state of membrane lipids following the change of growth temperature in *Yersinia enterocolitica*. *Microbiology and Immunology* **35**, 1085-1093.
76. **Nakashima K, Kanamaru K, Mizuno T, and Horikoshi K.** 1996. A novel member of the *cspA* family of genes that is induced by cold shock in *Escherichia coli*. *J Bacteriol* **178**: 2994-2997.
77. **Nashimoto H, and Nomura M.** 1970. Structure and function of bacterial ribosomes, XI. Dependence of 50S ribosomal assembly on simultaneous assembly of 30S subsunits. *Proc Natl Acad Sci USA* **67**: 1440-1447.
78. **Nashimoto H, Miura A, Saito H, and Uchida H.**1985. Suppressors of temperature-sensitive mutations in a ribosomal protein gene, rpsL (S12), of *Escherichia coli*. *Mol Gen Genet* **199**: 381-387.
79. **Neidhardt FC, Ingraham JL, and Schaechter M.** 1990. The effects of temperature, pressure and

pH. In: *Physiology of the bacterial cell, a molecular approach.* Sinauer, Sunderland, Mas, USA, pp 226-237.

80. **Olsen RH, and Metcalf ES.** 1968. Conversion of mesophilic to psychrophilic bacteria. *Science* **162**: 1288-1289.
81. **Oshima A, Fukunaga N, and Sasaki S.** 1987. Effect of temperature on natural mRNA-directed protein synthesis, psychrophilic and mesophilic bacteria. *J Fac Sci Hokkaido Univ Ser V (Botany)* **14**: 1-10.
82. **Panoff JM, Corroler D, Thammavongs B, and Boutibonnes P.** 1997. Differentiation between cold shock proteins and cold acclimation proteins in a mesophilic Gram-positive bacterium, *Enterococcus faecalis* JH2-2. *J Bacteriol* **179**: 4451-4454.
83. **Panoff JM, Legrand S, Thammavongs B, and Boutibonnes P.** 1994. The cold shock response in *Lactococcus lactis* subsp. *lactis*. *Curr Microbiol* **29**: 213-216.
84. **Phan-Thanh L, and Gormon T.** 1995. Analysis of heat and cold shock proteins in *Listeria* by two-dimensional electrophoresis. *Electrophoresis* **16**: 444-450.
85. **Potier P, Hipkiss AR, and Kushner DJ.** 1985. Protein turnover in a psychrotrophic bacterium. *Arch Microbiol* **142**: 28-33.
86. **Potier P, Drevet P, Gounot AM, and Hipkiss AR.** 1987. Protein turnover in a psychrotrophic bacterium: proteolytic activity in extracts of cells grown at different temperatures. *FEMS Microbiol Lett* **44**: 267-271.
87. **Potier P, Drevet P, Gounot AM, and Hipkiss AR.** 1990. Temperature dependent changes in proteolytic activities and protein composition in the psychrotrophic bacterium *Arthrobacter globiformis* SI55. *J Gen Microbiol* **136**: 283-291.
88. **Py B, Causton H, Mudd EA, and Higgins CF.** 1994. A protein complex mediating mRNA degradation in *Escherichia coli*. *Mol Microbiol* **14**: 717-729.
89. **Py B, Higgins CF, Krisch HM, and Carpousis AJ.** 1996. A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. *Nature* **381**: 169-172.
90. **Qoronfleh MW, Debouck C, and Keller J.** 1992. Identification and characterization of novel low-temperature-inducible promoters of *Escherichia coli*. *J Bacteriol* **174**, 7902-7909.
91. **Ray MK, Seshu Kumar G, and Shivaji S.** 1994a. Phosphorylation of membrane proteins in response to temperature in an Antarctic psychrotroph *Pseudomonas syringae*. *Microbiology* **140**: 3217-3223.
92. **Ray MK, Seshu Kumar G, and Shivaji S.** 1994b. Phosphorylation of lipopolysaccharides in the Antarctic psychrotroph *Pseudomonas syringae*: a possible role in temperature adaptation. *J Bacteriol* **176**: 4243-4249.
93. **Ray MK, Sitaramamma T, Ghandhi S, and Shivaji S.** 1994c. Occurrence and expression of *cspA*, a cold shock gene, in Antarctic psychrotrophic bacteria. *FEMS Microbiol Lett* **116**: 55-60.
94. **Roberts ME, and Inniss WE.** 1992. The synthesis of cold shock proteins and cold acclimation proteins in the psychrophilic bacterium *Aquaspirillum arcticum*. *Curr Microbiol* **25**: 275-278.
95. **Russell NJ.** 1984a. Mechanisms of thermal adaptation in bacteria: blueprints for survival. *Trends Biochem Sci* **9**: 108-112.
96. **Russell NJ.** 1984b. The regulation of membrane fluidity in bacteria by acyl chain length changes. In: *Biomembranes 12, Membrane fluidity.* Kates M, and Manson LA (eds). Plenum, New York, USA, pp. 329-347.
97. **Russell NJ.** 1990. Cold adaptation of micro-organisms. *Phil Trans R Soc Lond* B **316**: 595-611.
98. **Russell NJ.** 1992. Physiology and molecular biology of psychrophilic micro-organisms. In: *Molecular biology and biotechnology of extremophiles.* Herbert RA, Sharp RJ (eds).: Chapman & Hall, New York, USA, pp. 203-224.
99. **Sacerdot C, Vachon G, Laalami S, Morel-Deville F, Cenatiempo Y, and Grunberg-Manago M.** 1992. Both forms of translational initiation factor IF2 (α and ß) are required for maximal growth of *Escherichia coli*. *J Mol Biol* **225**: 67-80.
100. **Sakamoto T, Higashi S, Wada H, Murata N, Bryant DA.** 1997. Low-temperature-induced desaturation of fatty acids and expression of desaturase genes in the cyanobacterium *Synechococcus* sp. PCC7002. *FEMS Microbiol Lett* **152**: 313-320.

101. **Schauer AT, Carver DL, Bigelow B, Baron LS, and Friedman DI.** 1987. I N Antitermination system: functional analysis of phage interactions with the host NusA protein. *J Mol Biol* **194**: 679-690.
102. **Schnier J, and Isono K.** 1984. Insertion of IS1 into the *rpsE* gene for ribosomal protein S5 causes cold-sensitivity in *Escherichia coli*. *Mol Gen Genet* **195**: 364-366.
103. **Shaw MK, and Ingraham JL.** 1965. Fatty acid composition of *Escherichia coli* as a possible controlling factor of the minimal growth temperature. *J Bacteriol* **90**: 141-146.
104. **Shiba K, Ito K, and Yura T.** 1986. Suppressors of the *sec*Y24 mutation: identification and characterization of additional *ssy* genes in *Escherichia coli*. *J Bacteriol* **166**: 849-856.
105. **Shiba K, Ito K, Nakamura Y, Dondon J, and Grunberg-Manago M.** 1986. Altered translation initiation factor 2 in the cold-sensitive *ssy*G mutant affects protein export in *Escherichia coli*. *EMBO J* **5**: 3001-3006.
106. **Sprengart ML, Fuchs E, and Porter AG.** 1996. The downstream box: an efficient and independent initiation signal in *Escherichia coli*. *EMBO J* **15**: 665-674.
107. **Sun X, Griffith M, Pasternak JJ, and Glick BR.** 1995. Low temperature growth, freezing survival, and production of antifreeze protein by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. *Can J Microbiol* **41**: 776-784.
108. **Szer W. 1970.** Cell free protein synthesis at 0EC: an activating factor from ribosomes of a psychrophilic micro-organism. *Biochim Biophys Acta* **213**: 159-170.
109. **Tafelshtein EE, Golubinsky EP, and Maramovich AS.** 1998. Mechanism of temperature adaptation in *Yersinia pseudotuberculosis*. Poster presentation at the 7th Int Congress on Yersinia. *Ned Tijdsch Med Microbiol Supp II* **6**: 28.
110. **Tai PC, and Jackson H.** 1969. Mesophilic mutants of an obligate psychrophile, *Micrococcus cryophilus*. *Can J Microbiol* **15**: 1145-1150.
111. **Tai PC, Kessler DP, and Ingraham J.** 1969. Cold-sensitive mutations in *Salmonella typhimurium* which affect ribosome synthesis. *J Bacteriol* **97**: 1298-1304.
112. **Tanabe H, Goldstein J, Yang M, and Inouye M.** 1992. Identification of the promoter region of the *Escherichia coli* major cold-shock gene, *cspA*. *J Bacteriol* **174**: 3867-3873.
113. **Tasaka Y, Gombos Z, Nishiyama Y, Mohanty P, Ohba T, Ohki K, and Murata N.** 1996. Targeted mutagenesis of acyl-lipid desaturases in *Synechocystis*: evidence for the important role of polyunsaturated membrane lipids in growth, respiration and photosynthesis. *EMBO J* **15**: 6416-6425.
114. **Thieringer HA, Jones PG, and Inouye M.** 1998. Cold shock and adaptation. *BioEssays* **20**: 49-57.
115. **VanBogelen RA, and Neidhardt FC.** 1990. Ribosomes as sensors of heat and cold shock in *Escherichia coli*. *Proc Natl Acad Sci USA* **87**: 5589-5593.
116. **Wada H, and Murata N.** 1989. *Synechocystis* PCC6803 mutants defective in desaturation of fatty acids. *Plant Cell Physiol, Tokyo* **30**: 971-978.
117. **Wada H, and Murata N.** 1990. Temperature-induced changes in the fatty acid composition of the cyanobacterium *Synechocystis* PCC6803. *Plant Physiol* **92**: 1062-1069.
118. **Wada H, Gombos Z, and Murata N.** 1990. Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. *Nature* **347**: 200-203.
119. **Wada H, Gombos Z, Sakamoto T, and Murata N.** 1992. Genetic manipulation of the extent of desaturation of fatty acids in membrane lipids in the cyanobacterium *Synechocystis* PCC6803. *Plant Cell Physiol, Tokyo* **33**: 535-540.
120. **Wada H, Gombos Z, and Murata N.** 1994. Contribution of membrane lipids to the ability of the photosynthetic machinery to tolerate temperature stress. *Proc Natl Acad Sci* USA **91**: 4273-4277.
121. **Wang N, Yamanaka K, and Inouye M.** 1999. CspI, the ninth member of the CspA family of *Escherichia coli*, is induced upon cold-shock. *J Bacteriol* **181**: 1603-1609.
122. **Whyte LG, and Inniss WE.** 1992. Cold shock proteins and cold acclimation proteins in a psychrotrophic bacterium. *Can J Microbiol* **38**: 1281-1285.
123. **Willimsky G, Bang H, Fischer G, and Marahiel MA.** 1992. Characterization of *cspB*, a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperatures. *J Bacteriol* **174**:

6326-6335.

124. **Wolffe AP. 1994** Structural and functional properties of the evolutionarily ancient Y-box family of nucleic acid binding proteins. *BioEssays* **16**: 245-251.
125. **Yamanaka K, Fang L, and Inouye M.** 1998. The CspA family in *Escherichia coli*: multiple gene duplication for stress adaptation. *Mol Microbiol* **27**: 247-255.
126. **Yano R, and Yura T.** 1989. Suppression of the *Escherichia coli rpo*H opal mutation by ribosomes lacking S15 protein. *J Bacteriol* **171**: 1712-1717.
127. **Zheng W, and Kathariou S.** 1994. Transposon-induced mutants of *Listeria monocytogenes* incapable of growth at low temperature (4EC). *FEMS Microbiol Lett* **121**: 287-292.
128. **Zheng W, and Kathariou S.** 1995. Differentiation of epidemic-associated strains of *Listeria monocytogenes* by restriction fragment length polymorphism in a gene region essential for growth at low temperatures (4°C). *Appl Environ Microbiol* **61**: 4310-4314.

4

Detection & Identification

this chapter is based on the following publication:

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Digoxigenin-labelled *inv*- and *ail*-probes for the detection and identification of pathogenic *Yersinia enterocolitica* in clinical specimens and naturally contaminated pig samples

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ABSTRACT

A non-radioactive colony hybridization method has been developed for rapid detection of *Yersinia enterocolitica* in primary isolates, and for differentiation between pathogenic and non-pathogenic strains. The analysis is based on the previous observations [Miller et al. 1989] that the *inv*-locus is present in all *Yersinia* species, while the *ail*-gene is only found in pathogenic *Yersinia*. A panel of 132 *Y. enterocolitica* strains was tested for hybridization with digoxigenin-labelled *ail*-probes, and tested for in vitro invasiveness in a tissue culture invasion assay (TCI). The results of both the hybridization and TCI-test were in good agreement with pathogenicity phenotypes as indicated by serotyping. All 39 strains that were positive in the TCI-test were also positive by hybridization with *ail*, whereas 91 out of 93 strains that were negative in the TCI-test were also negative in hybridization with *ail*. Two strains appeared to hybridize with *ail* although they were negative in the TCI-test. The practicability of the method was investigated by testing 86 primary isolates of human, animal, or slaughterhouse origin for hybridization with *inv*- and/or *ail*-probes and comparison of the results with those of conventional methods to detect and identify *Y. enterocolitica*. *Yersinia* species and pathogenic *Yersinia* were detected in 19 and 16 samples, respectively, by cultivation and bio/serotyping. In hybridization with *ail*-probes, positive signals were obtained with 15 out of 16 samples in which pathogenic Yersinia had been detected (one sample was not tested). Hybridization with *inv*-probes was tested in four of the 19 samples from which *Yersinia* species had been isolated, and these also produced positive hybridization signals. On the other hand, *Yersinia* species could not be isolated from 67 samples, and 60 of these were negative in hybridizations with both *ail* and *inv*-probes. Hybridization results that were distinct from the results of cultivation were found only with slaughterhouse swab-samples, i.e. two out of 51 cultivation-negative samples produced strong positive signals with *ail* and/or *inv*, while five samples produced weak signals with one or both of the probes.

These results indicate that the described method can be used for (i) the identification of pathogenic *Y. enterocolitica* isolates and (ii) the detection of *Yersinia* species in primary isolates of naturally contaminated samples.

4.1 INTRODUCTION

'Yersiniosis' is a, mainly food-borne, gastro-intestinal human disease [Lee 1977; Black *et al.* 1978; Tacket *et al.* 1984; Tauxe *et al.* 1987] which is often accompanied by other, wide-ranging, clinical manifestations [Bottone 1977; Stern & Pierson 1979; Bos *et al.* 1985]. It is caused by pathogenic strains of *Yersinia enterocolitica*, which can be distinguished from non-pathogenic strains by *in vivo* virulence tests in animals, or on the basis of their serotype and/or biotype [Bottone 1977; Wauters 1981; Cornelis *et al.* 1987; Wauters *et al.* 1987]. As animal tests are less desirable and sero- and biotyping is time-consuming and not always reliable [Zink *et al.* 1982; Cornelis *et al.* 1987; Miller *et al.* 1989], alternative methods to distinguish between pathogenic and non-pathogenic strains have been sought.

Research on the genetic basis of the phenotypic differences between *Y. enterocolitica* serotypes has resulted in the discovery of a virulence-associated plasmid [Lee *et al.* 1977; Gemski *et al.* 1980] and the presence or absence of this plasmid has been used to discriminate between virulent and non-virulent strains [Bölin *et al.* 1982; Hill *et al.* 1983; Jagow & Hill 1986; Miliotis *et al.* 1989; Kapperud *et al.* 1990; Nesbakken *et al.* 1991]. However, methods based on the detection of the virulence plasmid may lead to false negative results, because the plasmid is easily lost during repeated subculturing of the bacteria [Zink *et al.* 1980; Portnoy *et al.* 1981; Portnoy & Falkow 1981]. More recently, plasmid independent properties were shown to be involved in *Y. enterocolitica* pathogenicity [Heesemann *et al.* 1984; Isberg & Falkow 1985; Isberg *et al.* 1987; Isberg 1989], especially adhesion and invasion features encoded by a chromosomal gene called *ail* (adhesion-invasion-locus) [Miller & Falkow 1988; Miller *et al.* 1990]. Hybridization with radioactive *ail*-probes and invasion into HEp-2 cells, as determined in an *in vitro* tissue culture invasion (TCI) assay, was shown to be restricted to strains of serotypes associated with disease [Miller *et al.* 1989]. Whereas *ail* was thus found uniquely in pathogenic strains of *Y. enterocolitica*, a second gene involved in invasion, designated *inv*, appeared to be specific for the whole genus *Yersinia* [Miller *et al.* 1989; Robins-Browne *et al.* 1989]. It has been suggested that *inv*-genes present in non-pathogenic strains were most likely not functioning [Pierson & Falkow 1990]. Based on these observations, *inv*-probes can be used to identify *Yersinia* species, and *in vitro* invasion (a TCI^+-phenotype) or the presence of *ail* can both be used to classify a particular *Y. enterocolitica* strain as pathogenic.

In this paper, we describe the development of a method based on digoxigenin (DIG) labelled *ail*- and *inv*-probes, which can be used to identify pathogenic and non-pathogenic *Yersinia* isolates. Since non-radioactive probes are used, the method is fit to detect virulent and non-virulent *Y. enterocolitica*, as a routine laboratory analysis, in primary cultures of environmental, human or animal origin. The sensitivity of DIG-labelled *ail*-probes was investigated by hybridizations using various types of DNA-blots. The specificity of the *ail*-probe was tested by hybridizations of culture dot-blots and colony blots from *Y. enterocolitica*

strains of 17 different serotypes. TCI-phenotypes of these strains were used to correlate hybridization results with potential for pathogenicity. The specificity of two *inv*-derived probes was tested on colony blots of a panel of bacterial species. Finally, *ail*- and *inv*-derived probes were used to detect, respectively, pathogenic *Y. enterocolitica* or *Yersinia* species in naturally contaminated samples, by means of colony blot hybridizations of primary cultures.

4.2 MATERIALS & METHODS

4.2.1 Bacterial Strains, Media, Growth Conditions

Yersinia enterocolitica, *Y. intermedia*, *Y. frederiksenii* and *Y. pseudotuberculosis* were sero- and biotyped collection-strains, isolated from human and non-human sources, from the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands. The strains were maintained at 4°C on nutrient agar slopes. For DNA-isolations, TCI-assays or blotting procedures, cultures were grown overnight at 30°C in nutrient broth or on Plate Count Agar (PCA, Oxoid), Tryptic Soy Broth (TSB, Oxoid) or Salmonella-Shigella-Deoxycholate-Calcium (SSDC, Oxoid) agar plates. *Escherichia coli* DH5α (BRL, Maryland, USA) competent cells and transformants were stored at -70°C in nutrient broth containing 25%(v/v) glycerol. For plasmid isolation, strains were grown at 37°C in nutrient broth supplemented with ampicillin (200 μg/ml). Type strains used to determine the specificity of the *inv*-probes were: *Listeria monocytogenes*, serotypes 1/2A (NCTC cat.no.7973) and 4B (NCTC cat.no.10527); *Escherichia coli* O:128 [Orskov *et al.* 1977]; *Salmonella typhimurium*, phage types 20 and 150 [Guinée *et al.* 1974]; and *Salmonella enteritidis*, phage types 1 and 2 (RIVM no.88-8859 and no.88-8993).

4.2.2 Plasmids & Probes

Standard DNA-techniques were used for DNA-isolations, restriction enzyme digestions, ligations, and plasmid transformations [Maniatis *et al.* 1982]. The restriction enzymes were obtained from Boehringer Mannheim (Germany). Plasmids pVM103 and pVM101, containing *Yersinia*-fragments with the *ail*- and *inv*-sequences, were kindly donated by Dr. V. Miller (University of California, Los Angeles, USA). Plasmid pVM103 was used to construct two plasmids which contained smaller *ail*-bearing Yersinia fragments, i.e. pRG101 and pRG102 (Figure 4.1).

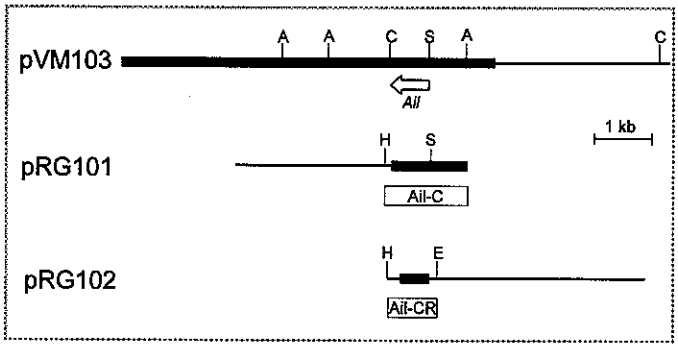

Figure 4.1
Plasmids and probes which harbour *ail*-sequences from *Y. enterocolitica*.
Bold lines: *Y. enterocolitica* DNA, thin lines: vector DNA. The position of the *ail*-gene is indicated by an arrow. Probes are depicted as open boxes. Relevant restriction sites are: A = *Ava*I, C = *Cla*I, E = *Eco*RI, H = *Hin*dIII, and S = *Sca*I.

Plasmid pRG101 was constructed by ligation of Ail-C, a 1.2 kb *Ava*I/*Cla*I fragment of pVM103 [Miller *et al.* 1989], into the *Ava*I/*Acc*I-linearized vector pUC19 [Yanisch-Perron *et al.* 1985]. Plasmid pRG102 was obtained by ligation of a 0.65 kb *Sca*I/*Hin*dIII fragment of pRG101 into the *Hin*dIII/*Sma*I-linearized vector pEMBL8 [Dente *et al.* 1983]. Fragments of pRG101 (*Ava*I/*Hin*dIII digest, 1.2 kb) and pRG102 (*Eco*R1/*Hin*dIII digest, 0.65 kb) were used to prepare the DIG-labelled probes Ail-C and Ail-CR, respectively. Both Ail-C and Ail-CR contained the complete *ail* gene. The probes differed in the length of the flanking sequence upstream of *ail*, i.e. 0.7 kb in Ail-C and 0.1 kb in Ail-CR [Miller *et al.* 1989; Miller *et al.* 1990]. Plasmid pVM101 was used to obtain probes Inv-D and Inv-B. Inv-D was prepared from the 3.6 kb *Cla*I-fragment of pVM101. This fragment had previously been designated Inv-ent [Miller & Falkow 1988] and had been used as a [^{32}P]-labelled probe for identification of *Yersinia* species The probe Inv-B was prepared from a 3.2 kb *Pvu*I/*Mlu*I-fragment of pVM101. Inv-B comprised almost the whole coding sequence of *inv*, plus a 0.7 kb sequence downstream of the gene [Young *et al.* 1990], whereas Inv-D contained only the 1.2 kb C-terminal part of the *inv*-gene, plus a 2.4 kb downstream sequence (Figure 4.2).

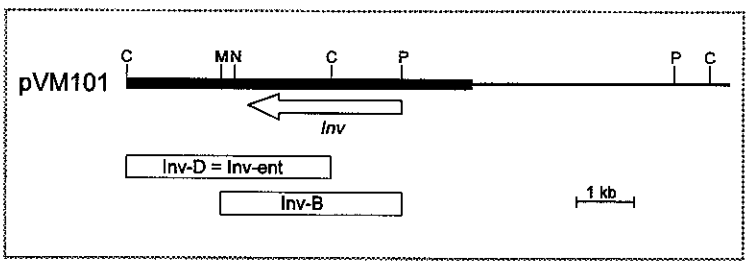

Figure 4.2
Plasmid and probes which harbour *inv*-sequences from *Y. enterocolitica*
Symbols as used in Figure 4.1 Relevant restriction sites are: C = *Cla*I, M = *Mlu*I, N = *Nco*I, and P = *Pvu*I.

All DNA-fragments were purified by means of gel-electrophoresis and treatment with 'GeneClean' (Bio101, La Jolla, Cal., USA), prior to labelling with the non-radioactive marker digoxigenin, applying a ready-to-use kit (DIG, Boehringer Mannheim, Germany) according to the manufacturer's instructions.

4.2.3 Sample Preparations

Human faeces

Approximately 1 g of sample was suspended in 2 ml of phosphate-buffered saline. Equal volumes (0.1 ml) were plated directly on Cefsulodin-Irgasan-Novobiocin agar (CIN, Oxoid) and plates were incubated for 48 or 96 h at 30°C. Duplicate plates were incubated at 22°C, which was meant to favour psychrotrophic *Yersinia*-like organisms [Schiemann 1982]. The remaining suspension was mixed with 25 ml of modified Rappaport-broth [Wauters 1973] and then split into two portions. One portion was incubated for 48 h at 30°C and the other one at 22°C. After this enrichment procedures, 0.1 ml volumes from both cultures were plated in duplicate onto Deoxycholate agar (DC, Oxoid) and further incubated for 48 h at both 30°C and 22°C. All plates showing bacterial growth were stored at 4°C, prior to subculturing and colony blotting.

Pig slaughterhouse samples

Various samples collected in a pig slaughterhouse (i.e. pig's tongues, contents of pig's ileum and rectum, swab-samples of carcasses, and swab-samples of machinery and employees hands') were investigated for the presence of *Yersinia* species by applying semi-cold enrichment in Irgasan-Ticarcillin-Potassiumchlorate (ITC) broth [Wauters *et al.* 1988], followed by incubation on selective CIN or SSDC agar [Boer *et al.* 1989]. All plates were stored at 4°C, prior to subculturing and colony blotting.

4.2.4 Subculturing and Identification

From all plates with primary isolates, colonies resembling *Yersinia* species on the basis of their morphological appearance (very small, colourless and translucent on SSDC; small, cream-coloured and with a red centre on CIN and DC) were subcultured. *Yersinia* strains were identified, serotyped and biotyped according to established criteria [Bercovier *et al.* 1980; Wauters 1981; Wauters *et al.* 1987].

4.2.5 Blotting Procedures and DNA-fixation

Southern blots with digested genomic DNA

Chromosomal DNA was isolated from *Y. enterocolitica* by means of proteinase-K/SDS lysis of the cells and subsequent CTAB/NaCl treatment and chloroform/isoamylalcohol extraction

of the lysate, as described elsewhere [Maniatis *et al.* 1982]. Portions of the DNA were digested to completion with either *Eco*RV, *Eco*RV+*Sca*I, *Sau*IIIA1 or *Sau*IIIA1+*Cfo*I, and the fragments were separated by agarose-gel electrophoresis. Southern blot transfer of the fragments on Hybond-N^+ membranes (Amersham/Life Sciences, Amersham, UK) was performed with {3.6 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA} blotting buffer, using a vacuum-blot apparatus (Millipore, Bedford, USA).

Spot-blots with purified total genomic DNA

DNA-concentrations of genomic and plasmid preparations were determined spectrophotometrically (OD_{260}/OD_{280}), and dilution series in 0.25 M NaOH were prepared containing either 0.005-10 ng/µl of plasmid pVM103, or 5-5000 ng/µl of purified genomic DNA. Equal volumes (1 µl) of the dilutions were pipetted in rows onto Genescreen-Plus membranes (Biotechnology Systems, 's-Hertogenbosch, the Netherlands) and on Hybond-N^+ membranes, producing well defined spots of immobilized DNA after air-drying .

Dot-blots with cell suspensions

Y. enterocolitica strains were grown overnight at 30°C, and the cell density was estimated spectrophotometrically (OD_{600}). Cells were pelleted by low speed centrifugation (5000 g, 10 min, 4°C). The supernatants were removed and the pellets were resuspended in sterile distilled water. From each suspension, a volume containing approximately 10^8 cells was mixed with NaOH to a final concentration of 0.25 M, and the cells were lysed by incubation at room temperature for 10 min. The lysates were transferred to a Genescreen-Plus membrane, using a 96-well dot-blot apparatus (Millipore, Bedford, USA), and the released DNA was fixed by air-drying of the membrane.

Colony blots

Small aliquots of freshly broth-cultured Yersinia reference strains and reference strains of other species were spotted onto agar plates and incubated overnight at 22°C or 37°C, to obtain distinct colonies on the plates. From these plates, as well as from those which had been obtained from clinical specimens and slaughterhouse samples, as described above, colonies were lifted onto circular membranes (Genescreen NEF-978X), according to the protocol provided by the manufacturer. Cell-lysis and simultaneous DNA-fixation was dône by alkaline steaming [Maas 1983]. Briefly, a hybridization membrane disc was placed onto the agar plate, left for 3 min, peeled off carefully, and transferred - with colony side up - to a piece of 3MM Whatman filter paper, that had previously been saturated with 0.5 M NaOH, in a pyrex petri dish. The dish (without lid) was placed in a steaming water bath for 10 min. Subsequently, the hybridization membrane was neutralized by soaking it (still with colony side up) on a pool of 0.75 ml 1M Tris-HCl buffer (pH=7.5). After 3 min, the neutralizing step was repeated once, with a fresh pool of buffer solution. Next, the membrane disc was transferred to {0.3 M NaCl, 0.03 M sodium citrate} wash buffer. Any excessive cellular debris was removed by rubbing

the membrane with a piece of wet paper towel. Finally, the rinsed membrane was air dried at room temperature.

4.2.6 Hybridization Conditions

Several preliminary experiments with blots of purified plasmid- and genomic DNA were performed, according to the hybridization- and detection protocol of the ready-to-use DIG-kit provided by the manufacturer. However, it was observed that, after finishing of the experiments, the stored GenescreenPlus membranes frequently attained, within a few days, an overall purple colouring, which overwhelmed the signals. Obviously, this colour development hindered the comparison of results of successive hybridizations. To eliminate this problem, we introduced several modifications in the composition of the hybridization solution.
Eventually, the modifications comprised the increase of the concentrations of both SDS and blocking agens (from 0.02 to 0.2% and from 0.5 to 2%, respectively), and the addition of heterologous DNA to a final concentration of 1µg/ml hybridization buffer. These modifications resulted in a better durability of finished membranes, a better signal-to-noise ratio, and a better reproducibility. For this reason, the modified hybridization solution was used in all subsequent experiments.

4.2.7 Tissue Culture Invasion Assay

The invasion capacity of all Yersinia strains was investigated by a tissue culture invasion (TCI-) assay, which was performed essentially as described by Falkow and co-workers [Miller & Falkow 1988]. HEp-2-cells used for this assay were obtained from Flow Laboratories (Rockville, Maryland, USA, cat. no. 0-26410).

4.3 RESULTS

4.3.1 Tissue Culture Invasion

TCI-phenotypes of two hundred European *Y. enterocolitica* strains of fifteen different serotypes were established, and the results are shown in Table 4.1. *In vitro* invasiveness (a TCI^+ phenotype) was found in all 26 strains of serotypes O:3 and O:9, which are commonly associated with disease. These results agreed with earlier findings for American strains [Miller *et al.* 1989] and supported the assumption that pathogenicity of *Y. enterocolitica* is coupled to invasiveness. With 42 serotype O:5 strains, both TCI-phenotypes were observed, and only two out of 132 strains of serotypes which are usually not associated with disease also showed a TCI^+ phenotype.

Table 4.1 Correlation between serotype, origin, *in vitro* invasiveness and DNA-hybridization with an *ail*-probe, of various strains of *Y. enterocolitica*.

SEROTYPES	ORIGIN		INVASIVENESS[1]			HYBRIDIZATION[2]	
	human	animal	+	-	nm	+	-
O:3	12	3	15	0	0	15	0
O:9	6	5	11	0	0	11	0
O:5	7	6	13	0	0	13	0
O:5	22	7	0	29	0	0	29
O:4,33	5	5	0	10	0	1	9
O:6,30	16	10	0	26	0	1	25
O:6,31	10	7	0	7	10	0	17
O:7,8	10	2	0	2	10	0	12
O:7,13a,b	11	5	0	4	12	0	16
O:10,34	6	5	0	3	8	0	11
O:12,25	2	1	0	2	1	0	3
O:14	3	0	0	0	3	0	3
O:16a,b	1	1	0	0	2	0	2
O:16a,58	5	10	0	1	14	0	15
O:27,41,43	3	2	0	3	2	0	5
O:40	2	0	0	2	0	0	2
O:41,43	3	0	0	3	0	0	3
O:47	1	6	0	1	6	0	7
Total number of strains	125	75	39	93	68	41	159
			132		68		
	200		200			200	

[1] In vitro invasiveness of strains, as measured in a Tissue Culture Invasion assay [Miller & Falkow 1988]: + = invasive, - = non-invasive, nm = not measured

[2] Signals obtained in culture dot-blot hybridization with a DIG-labelled *ail*-probe, as described in this paper: + = hybridization, - = no hybridization.

4.3.2 Sensitivity and Specificity of DIG-labelled Probes

Sensitivity

Southern blots of *Eco*RV-digested DNA from four TCI$^+$ strains and two TCI$^-$ strains were hybridized with Ail-C by the prescribed protocol. Single positive signals were obtained with 7.2 and 10.5 kb fragments from the TCI$^+$ strains of serotype O:9 and O:3, respectively, while no hybridization was observed with DNA from the TCI$^-$ strains (results not shown). These results were in agreement with those obtained with [^{32}P]-labelled *ail*-probes [Miller & Falkow 1988; Miller *et al.* 1989], and indicated that the sensitivity of the method using DIG-labelled probes was

sufficient to detect homologous fragments of DNA in Southern-blots. The detection limit for *ail* DNA was investigated with 1µl spot-blots of purified plasmid and genomic DNA, obtained from pVM103 and four different serotypes of *Y. enterocolitica*, respectively. When using the modified hybridization buffer, and probe Ail-C in the recommended concentrations (1-10 ng/ml), the lower detection limit of Ail-C homologous DNA, as present in pVM103, appeared to rank between 0.001 and 0.005 ng (Figure 4.3, upper row). Signals of similar intensity were obtained with spots containing ca. 25 ng of purified genomic TCI$^+$ *Y. enterocolitica* DNA, which originated from approximately $5x10^6$ cells (Figure 4.3, middle rows). In contrast, up to 1µg of TCI$^-$ DNA, originating from up to 10^9 cells, only produced background signals (Figure 4.3, lower row). No differences were found between the results obtained with Hybond-N$^+$, Genescreen-Plus or NEF-978X membranes (results not shown).

plasmid-derived DNA: Ail-C	1	0.5	0.1	0.05	0.01	0.005	0.001	0.0005
genomic DNA,TCI+, serotype O:9	5000	2500	500	250	50	25	5	2.5
genomic DNA,TCI+, serotype O:3	5000	2500	500	250	50	25	5	2.5
genomic DNA,TCI−, O:6,30	5000	2500	500	250	50	25	5	2.5

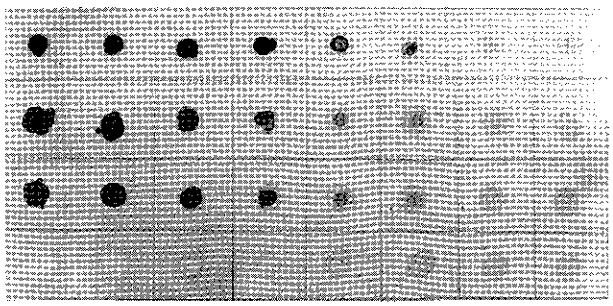

Figure 4.3
Inoculation scheme, and finished membrane, of a spot-blot-experiment. Spots contain 0.005-1 ng Ail-C DNA (as present in pVM103) or 2.5-5000 ng total genomic DNA of *Y. enterocolitica* (as purified from $5x10^5$ - 10^9 cells). The blot was hybridized with DIG-labelled Ail-C (1 ng/ml). Shaded inoculation sites are known, or presumed, to contain *ail* DNA

Specificity of *ail*-probes in culture-blots

Culture dot-blots of 200 strains were hybridized with Ail-C, and 61 of them also with Ail-CR. Figure 4.4 shows the inoculation scheme and the results of one of the membranes. The results are summarized in Table 4.1, and show that all TCI$^+$, but only two TCI$^-$, strains hybridized with the *ail*-probe. No differences in sensitivity or specificity were observed using either Ail-C or Ail-CR.